

LOAN DOCUMENT

DTIC ACCESSION NUMBER	PHOTOGRAPH THIS SHEET	INVENTORY
	LEVEL	
	<div style="border: 1px solid black; padding: 5px; margin: 10px auto; width: 80%;">AFRL-ML-TY-TR-1998-4536</div> <div style="text-align: center;">DOCUMENT IDENTIFICATION Aug 97</div>	
	<div style="border: 1px solid black; padding: 10px; margin: 10px auto; width: 80%;">DISTRIBUTION STATEMENT A Approved for public release; Distribution Unlimited</div>	
DISTRIBUTION STATEMENT		
DATE RECEIVED IN DTIC		
PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-FDAC		

DTIC ACCESSION NUMBER

LEVEL

INVENTORY

AFRL-ML-TY-TR-1998-4536

DOCUMENT IDENTIFICATION
Aug 97

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

DISTRIBUTION STATEMENT

ACCESSION FOR

NTIS	GRAM	<input checked="" type="checkbox"/>
DTIC	TRAC	<input type="checkbox"/>
UNANNOUNCED		<input type="checkbox"/>
JUSTIFICATION		

BY

DISTRIBUTION/

AVAILABILITY CODES

DISTRIBUTION	AVAILABILITY AND/OR SPECIAL
A-1	

DISTRIBUTION STAMP

DATE ACCESSIONED

DATE RETURNED

REGISTERED OR CERTIFIED NUMBER

19981015 079

H
A
N
D
L
E

W
I
T
H

C
A
R
E

AFRL-ML-TY-TR-1998-4536



**COMPENDIUM OF TECHNICAL PAPERS ON THE
REDUCTIVE DECHLORINATION OF CHLORINATED
SOLVENTS**

**GOSSETT, J.M.; ZINDER, S.H.; ET AL
CORNELL UNIVERSITY
SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
HOLLISTER HALL
ITHACA NY 14853-3501**

AUGUST 1997

Approved for Public Release: Distribution Unlimited.

**AIR FORCE RESEARCH LABORATORY
MATERIALS & MANUFACTURING DIRECTORATE
AIRBASE & ENVIRONMENTAL TECHNOLOGY DIVISION
TYNDALL AFB FL 32403-5323**

DTIC QUALITY INSPECTED 4

NOTICES

This technical paper contains several refereed journal articles related to the same topic. It is published as received and has not been edited by the technical editing staff nor the United States Air Force Research Laboratory, Materials and Manufacturing Directorate, Airbase and Environmental Division (AFRL/MLQE), Tyndall Air Force Base (AFB), Florida.

This paper documents work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any employees, nor any of their contractors, subcontractors, no contractor employees, make any warranty expressed or implied, or assume any legal liability or responsibility for the accuracy, completeness, or usefulness of any privately owned rights. Reference herein to any specific commercial products or process, by trade name, trademark, manufacturer, or otherwise does not necessarily constitute nor imply its endorsement, recommendation, or favoring by the United States Government or any agency, contractor, or subcontractor, thereof. The views and opinions of the authors expressed herein do not necessarily state nor reflect those of the United States Government of any agency, contractor, nor contractor thereof.

When Government drawings, specifications, or other data are used for any purpose other than in connection with a definitely Government-related procurement, the United States Government incurs no responsibility or any obligations, whatsoever. The fact that the Government may have formulated or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication, or otherwise in any manner construed, as licensing the holder or any other person or corporation; or as conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

This technical report has been reviewed by the Public Affairs Office (PA) and is releasable to the National Technical Information Service (NTIS) where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

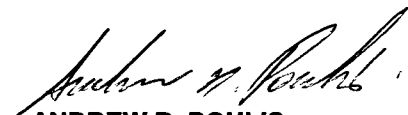
DESTRUCTION NOTICE: DESTROY BY ANY METHOD THAT WILL PREVENT DISCLOSURE OF CONTENTS OR RECONSTRUCTION OF THE DOCUMENT.



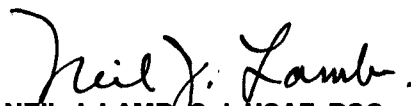
TIMOTHY G WILEY, Maj, USAF, BSC
Biosystems Area Manager



CHRISTINE WAGENER-HULME, LtCol, USAF, BSC
Chief, Environmental Technology Development Branch



ANDREW D. POULIS
Scientific & Technical Info Prog Mgr



NEIL J. LAMB, Col, USAF, BSC
Chief, Airbase & Environmental Technology Division

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 1997	3. REPORT TYPE AND DATES COVERED Final Report Aug 93 - Oct 96		
4. TITLE AND SUBTITLE Compendium of Technical Papers on the Reductive Dechlorination of Chlorinated Solvents			5. FUNDING NUMBERS	
6. AUTHOR(S) Multiple Authors				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cornell University School of Civil and Environmental Engineering Hollister Hall Ithaca, NY 14853-3501			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) AFRL/MLQE 139 Barnes Drive, Suite 2 Tyndall AFB FL 32403-5323			10. SPONSORING/MONITORING AGENCY REPORT NUMBER AFRL-ML-TY-TR-1998-4536	
11. SUPPLEMENTARY NOTES AFRL/MLQE Project Officer: Catherine M Vogel (850) 283-6208, DSN 523-6208				
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited. (PA Case # 98-415)			12b. DISTRIBUTION CODE A	
13. ABSTRACT (Maximum 200 words) This compendium of technical papers represents three years of work in the investigation of the anaerobic biodegradation of tetrachloroethylene (PCE). Cornell researchers had previously developed a methanol (MeOH)/PCE enrichment culture which dechlorinates high concentrations of PCE and other chlorinated ethenes to ethene (ETH), representing complete detoxification. This culture dechlorinates PCE at unprecedented, high rates with efficient use of MeOH as the electron donor for reductive dechlorination. However, research at Cornell showed that MeOH was not the direct donor for PCE dechlorination, but rather H ₂ . MeOH and other reductants found to support dechlorination merely serve as H ₂ precursors. Three alternative electron donors (ethanol, butyrate, and lactate) were evaluated to circumvent the problem of methanogenic competition for the supplied donor. The final selected substrate was used in a continuous-flow reactor study with the H ₂ /PCE enrichment culture. Engineering studies examined the kinetics of chlorinated ETH utilization, with emphasis on vinyl chloride (VC) dechlorination to ETH. Acclimation and induction issues were explored. Microbiological studies towards a better understanding of the nature and the requirements of the dechlorinating organisms were explored. The nutrition of the dechlorinating organisms was examined with the goal of finding and identifying reliable high-potency sources if the nutrients.				
14. SUBJECT TERMS Hydrogen donor, anaerobic reductive dechlorination, tetrachloroethene, anaerobic enrichment culture, methanogenesis, acetogenesis, trichloroethylene			15. NUMBER OF PAGES 42	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF REPORT Unclassified	19. SECURITY CLASSIFICATION OF REPORT Unclassified	20. LIMITATION OF ABSTRACT UL	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

CLASSIFIED BY:

DECLASSIFY ON:

TABLE OF CONTENTS

Section	Title	Page
1	Fennell, D. E., J. M. Gossett, and S. H. Zinder, "Comparison of Butyric Acid, Ethanol, Lactic Acid, and Propionic Acid as Hydrogen Donors for the Reductive Dechlorination of Tetrachloroethene," <i>Environmental Science & Technology</i> , 31:918-926 (1997)	1
2	Fennell, D. E., M. A. Stover, S. H. Zinder, and J. M. Gossett, "Comparison of Alternative Electron Donors to Sustain PCE Anaerobic Dechlorination," pages 9-16 in Hinchee, R. E., A. Leeson, and L. Semprini (eds.), <i>Bioremediation of Chlorinated Solvents</i> , Columbus, OH, Battelle Press (1995)	9
3	Maymón-Gatell, X., V. Tandoi, J. M. Gossett, and S. H. Zinder, "Characterization of an H ₂ -Utilizing Enrichment Culture That Reductively Dechlorinates Tetrachloroethene to Vinyl Chloride and Ethene in the Absence of Methanogenesis and Acetogenesis," <i>Applied and Environmental Microbiology</i> 61:3928-3933 (1995)	18
4	Smatlak, C. R., J. M. Gossett, and S. H. Zinder, "Comparative Kinetics of Hydrogen Utilization for Reductive Dechlorination of Tetrachloroethene and Methanogenesis in an Anaerobic Enrichment Culture," <i>Environmental Science & Technology</i> , 30:2850-2858 (1996)	24
5	Tandoi, V., T. D. Distefano, P. A. Bowser, J. M. Gossett, and S. H. Zinder, "Reductive Dehalogenation of Chlorinated Ethenes and Halogenated Ethanes by a High-Rate Anaerobic Enrichment Culture," <i>Environmental Science & Technology</i> , 28:973-979 (1994)	33
6	Zinder, S. H., and J. M. Gossett, "Reductive Dechlorination of Tetrachloroethene by a High Rate Anaerobic Microbial Consortium," <i>Environmental Health Perspectives</i> , 103 (5):5-7 (1995)	40

Comparison of Butyric Acid, Ethanol, Lactic Acid, and Propionic Acid as Hydrogen Donors for the Reductive Dechlorination of Tetrachloroethene

DONNA E. FENNELL AND
JAMES M. GOSSETT*

School of Civil and Environmental Engineering, Hollister Hall,
Cornell University, Ithaca, New York 14853

STEPHEN H. ZINDER

Section of Microbiology, Wing Hall, Cornell University,
Ithaca, New York 14853

Previous studies indicated that dechlorinators can utilize H_2 at lower concentrations than can methanogens. This suggests a strategy for selective enhancement of dechlorination—managing H_2 delivery so as to impart a competitive advantage to dechlorinators. Four H_2 donors—butyric and propionic acids, which can only be fermented when the H_2 partial pressure is lower than $10^{-3.5}$ or $10^{-4.4}$ atm, respectively, and ethanol and lactic acid, which are readily fermented at H_2 partial pressures 2–3 orders of magnitude higher—were administered to anaerobic mixed cultures. Comparison of the resulting enrichment cultures during time-intensive, short-term tests showed significant differences in patterns of donor degradation, H_2 production and use, and distribution of reduction equivalents between dechlorination and competing methanogenesis. Amendment with butyric and propionic acids resulted in less methanogenesis than did amendment with ethanol or lactic acid, which generated much higher H_2 levels. Ethanol did not support complete dechlorination during short-term tests, but it was a viable donor over long-term testing because a portion was converted to a pool of slowly degraded propionic acid and because during long-term tests, cultures were routinely co-amended with pre-fermented yeast extract, a source of slowly fermented volatile fatty acids. Understanding the fate of electron donors and their fermentation products is an important component in understanding dechlorinating communities.

Introduction

Tetrachloroethene (perchloroethylene, PCE) is a chlorinated solvent and suspected carcinogen that is also a common groundwater pollutant. Despite one preliminary report of aerobic degradation (1), the primary process by which PCE is transformed is through sequential reductive dechlorination under anaerobic conditions. Some organisms dechlorinate PCE slowly and incompletely in what is believed to be a co-metabolic process (2–6). Of greater interest for *in situ* bioremediation applications are the high rates of degradation that are exhibited by several newly isolated organisms that gain energy directly from dechlorination of PCE by using it as a respiratory electron acceptor. Reductive dechlorination

of PCE requires the addition of two electrons for each chlorine removed, and for three of the seven recently identified dechlorinating organisms, H_2 is one of the substrates (and in some cases, the only one) that can serve as the direct electron donor. The culture under investigation in our laboratories uses only H_2 as an electron donor and dechlorinates PCE completely to ethene (ETH) (7). *Dehalobacter restrictus* is another direct dechlorinator that uses only H_2 as an electron donor, but dechlorinates PCE only to *cis*-1,2-dichloroethene (*cis*DCE) (8, 9). *Dehalospirillum multivorans* also dechlorinates PCE to *cis*DCE using H_2 , but has a much more widely varied biochemical repertoire; it is additionally able to use various organic substrates such as pyruvate, lactate, ethanol, formate, and glycerol as electron donors (10–12). Other PCE-dechlorinating organisms have been isolated that do not use H_2 (13–15).

From the small but growing pool of knowledge about dechlorinating organisms, it thus appears that H_2 may serve an important role in reductive dechlorination of PCE in many environments. In natural systems, including contaminated aquifers, most H_2 becomes available to hydrogenotrophic microorganisms through the fermentation of more complex substrates by other members of the microbial consortium. The dechlorinators must then compete with other organisms, such as methanogens and sulfate-reducing bacteria, for the evolved H_2 . During studies in which ethanol or lactate was used to stimulate dechlorination in our mixed anaerobic enrichment culture, Stover (16) observed both active dechlorination and methanogenesis at high H_2 levels; however, when H_2 levels fell, dechlorination continued, albeit slowly, while methanogenesis ceased entirely. He speculated that the addition of electron donors that are fermented only under low H_2 partial pressures might give a selective advantage to dechlorinators over methanogens.

Smatlak et al. (17) later determined that the half-velocity constant with respect to H_2 for this dechlorinator was one-tenth that of the methanogenic organisms in the culture. The threshold H_2 level for dechlorination was also correspondingly lower than values typically reported for methanogens. Though confirmed thus far with only this one dechlorinator, there are thermodynamic reasons (i.e., the relatively high free energy available from dechlorination) to suspect that the threshold for H_2 use by dechlorinators may generally be lower than that for hydrogenotrophic methanogens (17). This suggests a strategy for selective enhancement of dechlorination—managing H_2 delivery so as to impart a competitive advantage to dechlorinators.

If substrates are added that degrade slowly and only under a low H_2 partial pressure—thus providing a slow and steady release of low levels of H_2 —dechlorination may be favored over competing methanogenesis. Substrates that are fermented to H_2 only under conditions of low H_2 include volatile fatty acids (VFAs) such as propionic and butyric acids (18–21). The Gibbs free energies for fermentation of these VFAs are actually positive under standard conditions (22), and microorganisms utilizing such fermentations for energy conservation are syntrophically dependent upon hydrogenotrophs to maintain H_2 partial pressures low enough to achieve negative free energies. The relationships between reaction free energies and H_2 partial pressures are illustrated in Figure 1 for the H_2 -yielding fermentations of interest to this study. In essence, because of thermodynamic limitations, propionate and butyrate are incapable of generating H_2 levels anywhere near the high levels possible with ethanol or lactate. The fermentations of butyric and propionic acids also generally proceed at slower rates than substrates that are exergonic at higher H_2 levels.

* Corresponding author telephone: 607-255-4170; fax: 607-255-9004; e-mail: jmg18@cornell.edu.

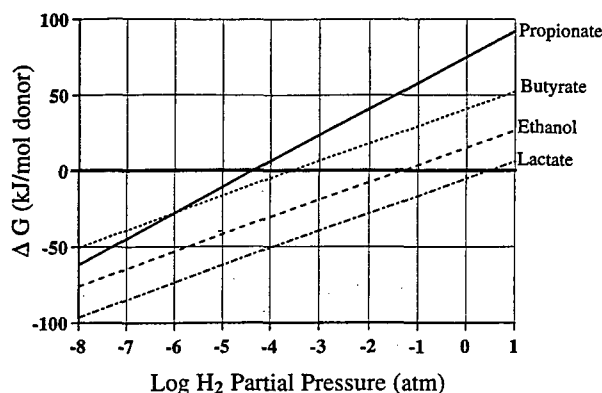


FIGURE 1. Effect of H₂ partial pressure on the free energy available from the fermentation of propionate, butyrate, ethanol, and lactate. Calculations were based on standard free energies and reactions in Thauer et al. (22) with temperature = 25 °C; pH = 7; HCO₃⁻ = 70 mM; propionate, butyrate, ethanol, and lactate concentrations = 0.5 mM; and acetate concentration = 5 mM.

The pairing of syntrophic propionate or butyrate fermenters and dechlorinators may form a biological system that will be naturally self-regulating—generating conditions of low level but steadily-produced H₂ to fuel dechlorination with minimal competition from methanogens. Conversely, the rapid fermentations of substrates such as ethanol and lactate that are exergonic at much higher H₂ levels will produce a short-lived “burst” of H₂, which will equally stimulate all the competing hydrogenotrophic organisms. Such conditions will result in an ever-growing pool of hydrogenotrophs—most of which do not contribute to the biodegradation of PCE, but which consume an ever greater portion of the added electron donor.

Numerous microcosm and site studies have shown successful stimulation of dechlorination with substrates such as methanol (23), ethanol (24, 25), lactate, butyrate (24–26), and benzoate (27, 28). However, understanding both the fate of the electron donors and the fate of H₂ evolved from their degradation and the extent to which their reducing equivalents are channeled to desirable dechlorination or competing H₂ sinks has important implications for determining how best to effectively stimulate latent dechlorinating activity for *in situ* anaerobic bioremediation and for analyzing and predicting results observed at naturally attenuated sites.

In this study, we documented the fate of electron donors added to an anaerobic, mixed dechlorinating culture and analyzed the resulting patterns of H₂ production/consumption and the distribution of H₂ use between competing processes of dechlorination and methanogenesis. We chose two substrates that are readily fermented under relatively high H₂ levels—ethanol and lactic acid—and two that are fermented only under low H₂ levels—butyric and propionic acids. Mixed anaerobic cultures were amended semi-continuously with one of the four donors for long-term determination of their relative abilities to maintain PCE dechlorination. Time-intensive studies were performed intermittently to give a more detailed picture of comparative culture performance, during which the biodegradation of electron donor and the formation of dechlorination products, H₂, and methane were intensively documented.

Materials and Methods

Chemicals and Stock Solutions. Butyric acid (Aldrich Chemical Co., 99%), ethanol (campus supplier, 95%), lactic acid (Fisher Scientific Co., 87.6%), propionic acid (Eastman Kodak Co., 99%), and PCE (Eastman Kodak Co., 99.9%) were routinely used as direct culture amendments and for preparation of analytical standards. Glacial acetic acid (Mallinckrodt, Inc., 99.5–100.5%), isobutyric acid (Fisher Scientific Co.,

99%), isovaleric acid (Aldrich Chemical Co., 99%), and hexanoic acid (Aldrich Chemical Co., 99.5%) were used for preparation of analytical standards and, during one experiment, for preparation of a surrogate fermented yeast extract. TCE (Fisher Scientific Co., 99%), VC (Matheson Gas Products, 99%), ETH (Matheson Gas Products), methane (Scott Specialty Gases), and H₂ (Airco, 1%, and Matheson Gas Products, pure, UHP) were used for preparation of analytical standards.

H₂SO₄ (Fisher Scientific Co., 95.9%) and H₃PO₄ (Mallinckrodt, Inc., 95.9%) were used to prepare 6 N and 8 N solutions, respectively, for preservation of liquid samples. Acidification of samples was at a rate of 10 μL per prefiltered 0.5 mL of sample.

Preparation of the basal salts medium (29) and PCE-laden basal salts medium containing approximately 1200 μM PCE (17) has been described elsewhere.

Pre-fermented yeast extract (FYE), a required nutritional supplement (30), was added to cultures at a rate of 20 μL (for 1:1 donor:PCE ratios) or 40 μL (for 2:1 donor:PCE ratios) per 100 mL of culture at each feeding. The concentration of reducing equivalents available in FYE was determined as follows: Culture bottles were fed FYE and PCE and were monitored to determine the total amount of equivalents (from FYE and endogenous decay) channeled to dechlorination and methanogenesis over 48 h. Other culture bottles were fed only PCE to determine the amount of equivalents from endogenous decay alone. The number of equivalents available from FYE was determined to be approximately 30 μequiv per 40 μL of FYE fed per 48 h. Seventy percent of the equivalents were accounted for by the contributions from measured concentrations of VFAs in FYE: propionic, butyric, isobutyric, isovaleric, and hexanoic acids.

Surrogate FYE (SFYE)—which was used in some experiments to replace the electron-donating capacity of the FYE—was an anoxic, aqueous mixture of reagent-grade, individual VFAs, each present at its measured FYE concentration. However, since 30% of the reducing equivalents in FYE were unaccounted for by VFA analysis, this difference was made up by employing additional butyric acid. The final VFA content of SFYE was 94 mM acetic acid, 18 mM propionic acid, 125 mM butyric acid, 6.6 mM isobutyric acid, 10.6 mM isovaleric acid, and 7.5 mM hexanoic acid. SFYE, where employed, was also added at a rate of 40 μL per 100 mL of culture.

A vitamin solution (7) was added at a rate of 0.5 mL/100 mL to previously unamended cultures and, thereafter, at a rate of 50 μL/10 mL fresh basal medium added.

Methanol-Enriched/High-PCE Source Culture. The source culture for all experiments was one enriched with methanol, PCE, and yeast extract (31). It was operated at a nominal PCE concentration of 550 μM—an inhibitory concentration for methanogenesis. Little methanogenic activity—including acetotrophic methanogenesis—was observed in this culture. This and all other cultures described were operated with a nominal hydraulic retention time of 40 days at 35 °C.

Since all experiments described here used PCE doses of 110 μM (one-fifth the level employed with the source culture), a 20% dilution of the source culture was used as starting material. The lack of acetotrophic activity made this culture especially suitable for these experiments. Fermentation of the added electron donors proceeded to acetic acid and H₂. With no conversion of acetic acid to methane, we could easily account for all H₂ equivalents that were channeled to methane formation. Without the inhibitory PCE concentrations, however, acetotrophic activity was expected to begin eventually. Unfortunately, it did occur after about 90 days, and after this time, a strict accounting of methane formed from H₂ was not possible.

Preparation of Culture for Serum Bottle Studies. Serum bottle tests were performed in 160-mL bottles with 100 mL

TABLE 1. Protocol for Long-Term Operation of Cultures for Comparison of Electron Donors at 1:1 and 2:1 Ratios of Donor:PCE

culture bottle set	electron donor	PCE added ^a (μ mol)	electron donor added ^a (μ mol)	electron donor added ^a (μ equiv) ^b
1:1 Ratio				
methanogenic cultures	H ₂	0	44	88
dechlorinating cultures and dechlorinating/methanogenic mixtures	butyric acid	11	22	88
	ethanol	11	22	88
	lactic acid	11	22	88
	propionic acid	11	14.7	88
	FYE only	11		$\approx 15^c$
2:1 Ratio				
dechlorinating/methanogenic mixtures	butyric acid	11	44	176
	ethanol	11	44	176
	lactic acid	11	44	176
	propionic acid	11	29.4	176
	FYE only	11		$\approx 30^c$

^a Indicated quantities were repetitively added every 2 days. ^b Assumptions for computation of equivalents (μ equiv/ μ mol): PCE, 8; H₂, 2; butyric acid, 4; ethanol, 4; lactic acid, 4; and propionic acid, 6. ^c The amount of reducing equivalents available from FYE was determined by observation of the amount of methane and dechlorination products formed.

of a 20% dilution of the source culture. Dilutions were prepared by transferring required volumes of methanol-enriched source culture and basal medium in purged, sealed bottles to an anaerobic glovebox and mixing these together in bulk to prepare a 20% dilution. The mixture was dispensed via a 100-mL volumetric pipette to 160-mL serum bottles. The serum bottles were capped with previously autoclaved, gray-butyl, Teflon-lined septa (Wheaton) and crimped with aluminum caps. Upon removal from the glovebox, the caps were removed as a cannula emitting a stream of 30% CO₂ and 70% N₂ purge gas was introduced. Each bottle was purged for 2 min to remove H₂—a potential electron donor that was present in the glovebox atmosphere—then were re-capped. Bottles were then fed according to the protocol shown in Table 1, and this setup day was defined as day 0. Bottles were routinely incubated in an inverted position on an orbital platform shaker (for long-term studies) or in an orbital shaker water bath (for time-intensive studies).

Protocol for Long-Term Operation of Serum Bottles. Every second day during long-term operation, the headspace of each bottle was sampled for dechlorination products, H₂, and methane; then PCE, electron donor (if any), and FYE were added. As will be shown later, because of its slow rate of degradation, propionic acid was sometimes withheld to avoid its accumulation in the cultures. Every fourth day, after headspace samples were analyzed, 10 mL of culture was removed via a purged, gas-tight syringe (Dynatech Precision Sampling Co.), 10 mL of fresh basal medium was added in the same manner, the septum and crimp cap were removed, and the bottle was purged for 5 min and then re-capped using a fresh septum. The removed liquid was used for measurement of pH, VFAs, ethanol, and lactic acid. After the exchange and purge, the bottles were fed PCE, electron donor (if any), FYE, and vitamin solution. pH of the cultures was typically 7–7.3.

Protocol for Time-Intensive Studies in Serum Bottles. After headspace samples were analyzed, 10 mL of culture was removed; the bottles were purged for 5 min and then re-capped with a fresh septum. Approximately 11 μ mol of pre-dissolved PCE was delivered by adding 9 mL of PCE-saturated basal medium and 1 mL of regular basal medium. Excess gas pressure of 7 mL was allowed to remain in each bottle to facilitate the removal of the gas and liquid samples without resulting in vacuum—and danger of introduction of air—in the bottle. After vitamin solution was added, the electron donor and any additional supplements were injected (time = 0). Except where noted, FYE was not added during the time-intensive studies for the comparison of electron donors to allow more accurate determination of the fate of

reduction equivalents provided by donor alone. Headspaces were analyzed intensively for approximately 10–48 h, and six to eight liquid samples were removed during the test to monitor electron donor degradation.

Comparison of the Electron Donors Butyric Acid, Ethanol, Lactic Acid, and Propionic Acid. Initially, bottles were operated with a 1:1 ratio of electron donor to PCE on an electron equivalent basis. Half of the bottles were fed only H₂ in order to ensure a healthy methanogenic population, and half were operated with one of the organic electron donors and PCE to ensure a healthy, dechlorinating population. A control bottle that received only FYE and PCE (but no other electron donor) was run to monitor the electron-donating capacity of the FYE. On days 36, 40, or 52, one or more of the dechlorinating cultures was subjected to time-intensive studies. After day 52 when the time-intensive studies were completed, each of the dechlorinating cultures was combined with one of the H₂-fed cultures, mixed, and re-distributed to duplicate bottles. By mixing a dechlorinating culture fed PCE with an H₂-fed, methanogenic culture, we could make certain that any perceived advantage (i.e., low levels of methanogenesis) was a result of advantageous patterns of H₂ production and not because of an unhealthy methanogenic population. Table 1 shows the protocols for long-term operation. The duplicate bottles were continued with the 1:1 ratio protocol for an additional 30 days. On day 80, one of the duplicates was switched to a 2:1 ratio of electron donor to PCE, the run continued for several weeks, and then time-intensive studies were performed on the 2:1 bottles.

Time-Intensive Study of Ethanol Supplemented with FYE or SFYE. Time-intensive studies were performed with duplicate cultures amended with ethanol only, ethanol plus FYE, or ethanol plus SFYE to determine the role of FYE as a slow electron donor in the success of long-term operations. At setup and then 2 days later, cultures were fed 11 μ mol of PCE, 88 μ mol of H₂, and FYE to ensure healthy cultures and to determine that both dechlorinators and methanogens were active. After 4 days the experiment was begun (defined as day 0). Thereafter, the cultures were fed (according to the long-term protocol) PCE (11 μ mol), ethanol (44 μ mol), vitamins, and FYE. The bottles were operated for 30 days before the time-intensive tests were performed.

Butyric Acid Enrichment Culture Development. A 6-L butyric acid enrichment culture fed a 2:1 electron donor:PCE ratio was developed using 10% inoculum from the methanol source culture. The operational protocol for the butyric acid-enriched culture has been described in detail elsewhere (17).

Analytical Methods. A gas chromatography system consisting of packed columns, two flame ionization detectors

(FID), a reduction gas detector (RGD), a thermal conductivity detector (TCD), and switching valves to divert constituents to specific detectors was used to quantify PCE, TCE, VC, ETH, methane, and H_2 (29). A single 0.1-mL headspace sample injection via a gas-tight syringe was used to obtain measurements of all the components when the H_2 level was within the detection limits of the RGD. Early in the experimentation, measurements of higher H_2 levels were made using the TCD method described by Smatlak et al. (17). Later, the TCD was placed in the carrier flow line prior to the RGD, and during periods when H_2 was above the detection range of the RGD, all measurements were obtained with a single 0.5-mL injection, while when H_2 levels were low—i.e., within the detection sensitivity of the RGD—a single 0.1-mL injection was used.

A Perkin-Elmer Corporation autosystem gas chromatograph with a 0.53-mm Nukol capillary column (Supelco, Inc.) and an FID was used for analysis of ethanol and VFAs. The N_2 carrier gas flow rate was 10 mL/min, the injector temperature was 200 °C, and the detector temperature was 250 °C. For VFA analysis, a 0.5- μ L sample was injected onto the column that was held at 90 °C for 9 min. For ethanol analysis, a 3- μ L sample was injected onto the column that was held isothermally at 70 °C for 1 min. Culture samples of 0.25 or 0.5 mL for ethanol and VFA analysis were filtered with a syringe filter (0.2 or 0.45 μ m Gelman) and preserved with 8 N H_3PO_4 prior to analysis.

Lactic acid (and the VFA content of time-intensive study samples from lactic acid-fed cultures) was determined with a Hewlett Packard 1090 high-performance liquid chromatograph (HPLC) with either a 300-mm HPX-87H ion-exclusion column operated at 65 °C or a 100-mm by 7.8-mm Fast-Acid column operated at ambient temperature (Bio-Rad Laboratories) and a diode-array detector at 210 nm. The mobile phase was 0.013 N H_2SO_4 , at 0.65 mL/min for the HPX-87H column and 0.7 mL/min for the Fast-Acid column. Samples of 0.5 mL were filtered and preserved with 6 N H_2SO_4 prior to analysis. The injection volume was either 60 or 100 μ L.

Results

Long-Term Comparison of Electron Donors. Results of the long-term operation were not significantly different among electron donors, in terms of the amount and extent of dechlorination. All donors facilitated dechlorination to VC and ETH in comparable amounts. Furthermore, increasing the electron donor:PCE ratio from 1:1 to 2:1 resulted in the production of more ETH in all cultures. Duplicate bottles behaved similarly. Consequently, only long-term results for ethanol-fed cultures are shown in detail (Figure 2).

Figure 2a shows the dechlorination product formation for an ethanol-fed culture. The saw-tooth configuration of the graph results from the depiction of the cumulative fate of the 11 μ mol of PCE added every second day. Since bottles were purged only every fourth day, every other data point depicts the dechlorination products of the total 22 μ mol of PCE that had been added up to that time. The ethanol culture was operated at a 1:1 ratio of electron donor to PCE for the first 80 days and at a 2:1 ratio thereafter. Added PCE was dechlorinated to VC and ETH except during day 36 to day 64 when PCE and TCE remained. This period of poor dechlorination was at first thought to be an indication of selective advantage to the methanogens—caused by high H_2 levels. However, the cultures eventually recovered completely, and we now believe that this temporary failure was the result of the upsetting nature of time-intensive studies that were performed on days 36 and 40. On day 52, the culture was blended with its H_2 -fed methanogenic counterpart. After blending, there was no significant increase in methanogenesis (Figure 2b) over that which had already been observed. After day 84, however, a significant increase in methanogenesis was observed and was caused by the onset of acetotrophic

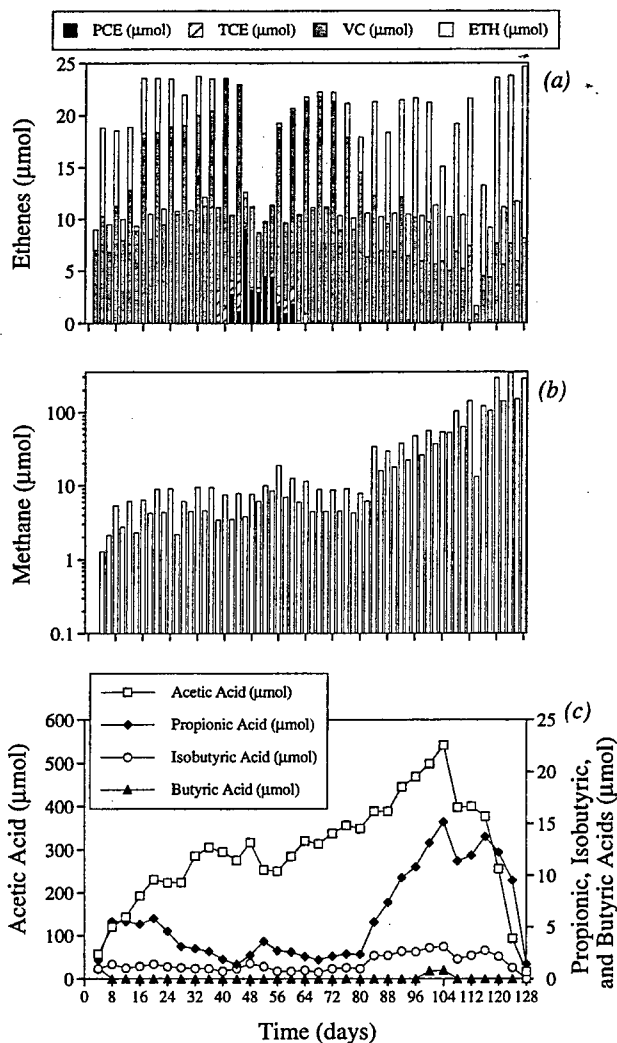


FIGURE 2. Long-term operation of ethanol-fed dechlorinating culture—fed 1:1 donor:PCE through day 80, blended with methanogenic counterpart day 52, and fed 2:1 donor:PCE after day 80: (a) dechlorination products, (b) methane production, and (c) VFAs.

activity. Acetic acid, which had accumulated from the degradation of ethanol, was rapidly degraded after day 100 as acetotrophic activity became significant (Figure 2c). An interesting aspect of ethanol degradation was the accumulation of propionic acid after ethanol loading was increased to a 2:1 donor:PCE ratio (Figure 2c). The accumulation of propionic acid in ethanol-fed microcosms has also been observed in our laboratory (32).

Lactic acid supported somewhat better dechlorination than ethanol (data not shown). Methane production developed more slowly than in ethanol-fed cultures, but eventually reached the same level (about 6 μ mol per feeding). Dechlorination was excellent after the increase in donor:PCE from a 1:1 to a 2:1 ratio. The VFA profile of this donor—like that of ethanol—yielded interesting results. Propionic acid accumulated in the bottles at startup and then again after the donor addition was increased beyond day 80.

Culture development on propionic acid apparently *did* have an exclusionary effect on the development of a methanogenic population (data not shown). Prior to blending with its methanogenic counterpart, only trace amounts of methane were produced. After blending, methane production was still only about one-fourth that of cultures amended with either ethanol or lactic acid. After day 80 when propionic acid addition was increased to a 2:1 donor:PCE ratio, methane production began to increase and dechlorination improved. Toward the end of the test, this methane increase was probably

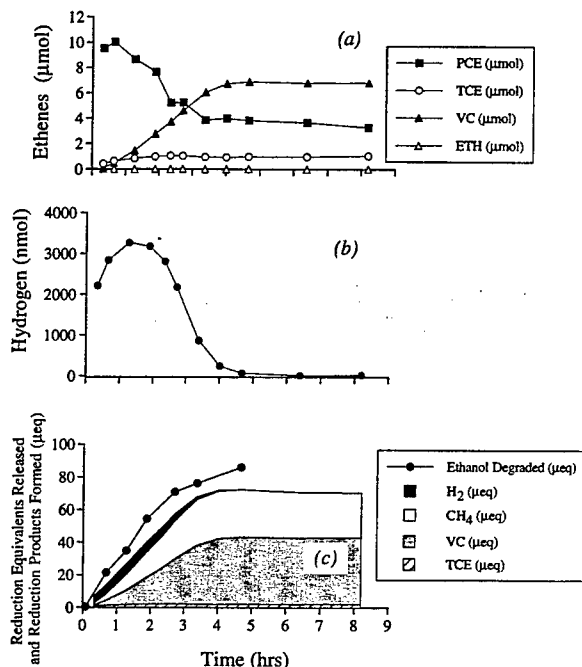


FIGURE 3. Time-intensive study of ethanol-fed culture at a 1:1 donor:PCE ratio (performed on day 36 of long-term operation): (a) dechlorination, (b) hydrogen, and (c) reduction products.

also associated with the onset of acetotrophic activity. Propionic acid was degraded slowly and was not completely consumed within the 2-day period between additions, but tended to accumulate. The slow degradation rate did not allow us to precisely double the electron donor:PCE ratio from 1:1 to 2:1, since we were sometimes forced to withhold it to avoid a significant increase above the desired level of amendment. Prior to time-intensive studies, for example, propionic acid was withheld so that it would be depleted. The depletion of propionic acid resulted in residual PCE on several occasions.

Butyric acid also served as an excellent donor over the long term (data not shown). It was degraded more readily than propionic acid and did not accumulate in bottles. The methane production in butyric acid-amended cultures was approximately 4 μmol per feeding.

The control bottles, which were amended with FYE but no other electron donor, exhibited trace amounts of methane formation and incomplete dechlorination with significant amounts of remaining PCE and TCE. The electron donating capacity of the FYE is shown in Table 1.

Time-Intensive Studies Comparing Electron Donors. While the comparison of electron donors over the long-term showed little difference in terms of the final dechlorination results, time-intensive studies did show marked differences among the donors. Results of time-intensive studies with ethanol at a 1:1 donor:PCE ratio (Figure 3) or 2:1 donor:PCE ratio (complete data set not shown) yielded a typical pattern. Dechlorination proceeded rapidly for the first 3.5 h (Figure 3a) and then slowed drastically. Methane was also produced rapidly during the initial 3.5 h and then production ceased (Figure 3c). H_2 production occurred in a burst of 3000–5000 nmol ($10^{-2.9}$ – $10^{-2.7}$ atm) within 2 h (Figure 3b, Figure 7) as the ethanol was rapidly degraded (Figure 3c).

The results from lactic acid time-intensive studies differed depending upon whether the donor was fed at a 1:1 donor:PCE ratio (Figure 4) or a 2:1 donor:PCE ratio. At a 1:1 ratio, degradation of lactic acid produced a peak of H_2 up to only 250 nmol (10^{-4} atm) (Figure 4b). At a 2:1 ratio the peak was 3000 nmol ($10^{-2.9}$ atm) (Figure 7). PCE dechlorination (Figure 4a) occurred rapidly while lactic acid was degraded and H_2 was being produced (Figure 4b), and it continued at a reduced

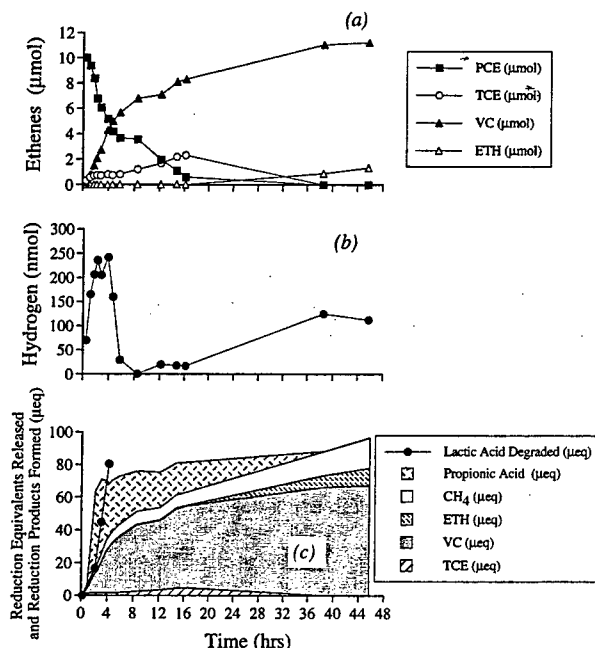


FIGURE 4. Time-intensive study of lactic acid-fed culture at a 1:1 donor:PCE ratio (performed on day 36 of long-term operation): (a) dechlorination, (b) hydrogen, and (c) reduction products.

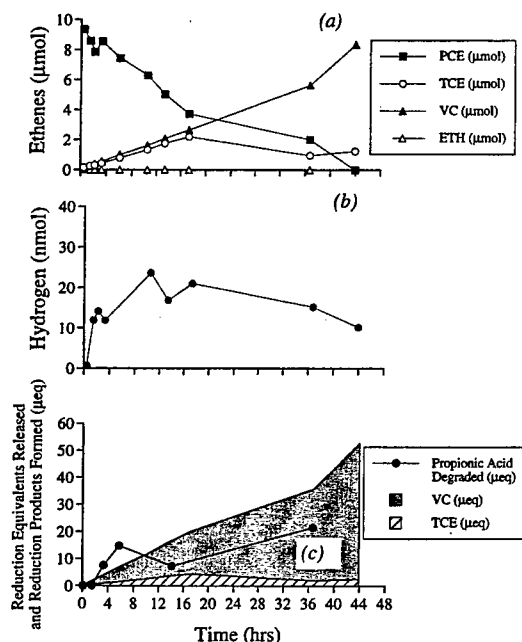


FIGURE 5. Time-intensive study of propionic acid-fed culture at a 1:1 donor:PCE ratio (performed on day 36 of long-term operation): (a) dechlorination, (b) hydrogen, and (c) reduction products.

rate after lactic acid was depleted. The continued dechlorination was probably stimulated by the presence of a significant pool of propionic acid (Figure 4c), which was apparently produced during lactic acid fermentation and then slowly degraded after lactic acid was depleted.

Results of a time-intensive study of propionic acid at a 1:1 donor:PCE ratio (Figure 5) produced a much different profile than with ethanol or lactic acid. PCE was slowly but steadily dechlorinated to VC over the 44-h period of testing (Figure 5a), and some TCE accumulated. Only a trace amount of methane was detected, and this was undoubtedly the result of the very low H_2 levels (20 nmol, $10^{-5.1}$ atm) that were maintained (Figure 5b). A graph of reduction products formed (Figure 5c) is also indicative of the nearly complete channeling of reduction equivalents to dechlorination (methane was too

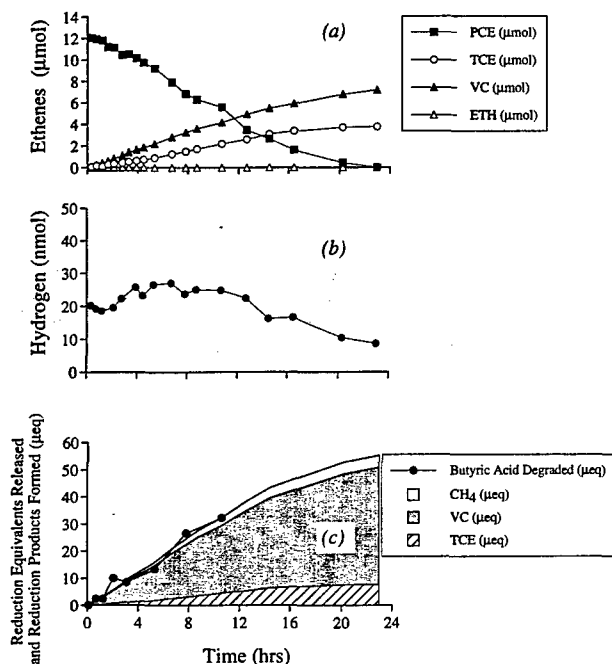


FIGURE 6. Time-intensive study of butyric acid-fed culture at a 1:1 donor:PCE ratio (performed on day 52 of long-term operation): (a) dechlorination, (b) hydrogen, and (c) reduction products.

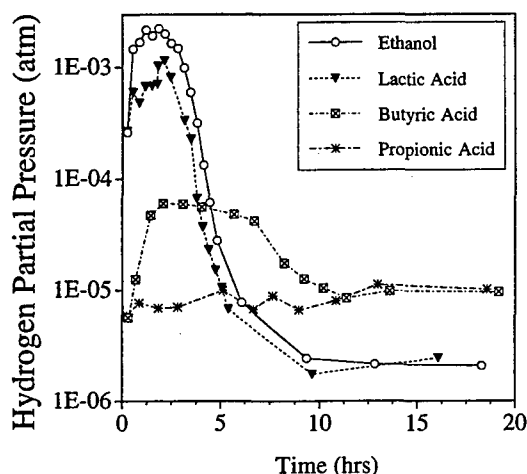


FIGURE 7. Comparison of hydrogen production from the degradation of ethanol, lactic acid, butyric acid, and propionic acid at 2:1 ratios of donor:PCE.

low to be evident on the graph). Patterns were very similar at 2:1 donor:PCE ratio (see H_2 data, Figure 7); however, because of the onset of acetotrophic methanogenesis, no methane production pattern from H_2 alone could be obtained.

Figure 6 shows results from a time-intensive study of butyric acid at a 1:1 donor:PCE ratio. Dechlorination proceeded steadily as PCE was converted to TCE and VC (Figure 6a). While significant quantities of TCE were observed during the time-intensive test, it was not detected at the end of a 48-h period during long-term studies. A steady H_2 level of 30 nmol (10^{-5} atm) or less was maintained (Figure 6b). Less methane was produced than in ethanol-fed cultures, but more than with propionic acid (Figure 6c). Addition of 2:1 butyric-acid:PCE resulted in a small accumulation of H_2 to 150 nmol ($10^{-4.2}$ atm) (Figure 7) and TCE was not a prominent intermediate.

Figure 7 shows a comparison of the H_2 levels produced by each of the electron donors at a 2:1 donor:PCE ratio. Peak H_2 levels from propionic acid, butyric acid, ethanol, and lactic acid were approximately $10^{-5.1}$, $10^{-4.2}$, $10^{-2.7}$, and $10^{-2.9}$ atm, respectively. These relative peak levels are roughly in

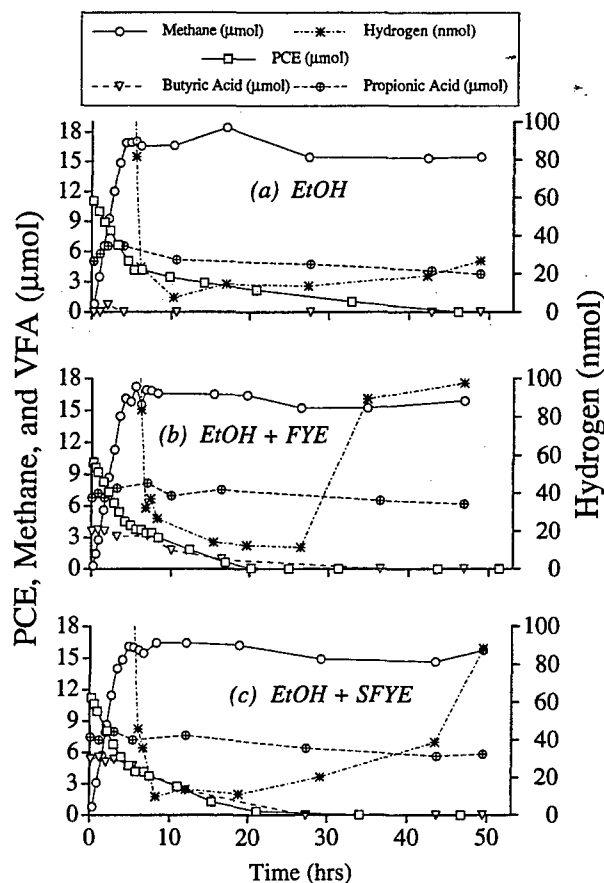


FIGURE 8. Time-intensive studies performed with ethanol-fed cultures at a 2:1 donor:PCE ratio with nutritional and electron donor supplements.

accordance with expectations from thermodynamic considerations (Figure 1). Thermodynamic upper limits to H_2 —i.e., H_2 levels causing each of the four fermentations to yield zero free energy—were estimated to be $10^{-4.4}$, $10^{-3.5}$, $10^{-1.2}$, and $10^{+0.5}$ atm, respectively. That the experimentally observed H_2 peaks were below thermodynamic limits is not surprising. In the first place, physiological upper H_2 limits must provide some finite free energy to the fermenting organisms. Secondly, the observed peak H_2 levels represent dynamic steady-state conditions where rates of H_2 production and use were balanced; therefore, rates of H_2 production were non-zero, unlike the situation at the physiological limit.

Role of FYE as a Slow Source of H_2 . FYE was routinely added to all the cultures as a nutritional supplement (7) during long-term operation but was omitted during the time-intensive studies. We believe that the addition of FYE greatly influenced the outcome of the long-term tests, but we were not certain if the difference was simply nutritional or—the more likely explanation—that a fraction of the FYE served as slowly available electron donor that fueled dechlorination after the initial burst from donor degradation, effectively masking the expected differences between the more slowly degraded H_2 sources that produce low levels of H_2 and those that are degraded more rapidly and produce much higher H_2 levels.

We therefore explored this issue in a series of time-intensive studies using ethanol, which generated a high-level H_2 -production pattern that resulted in incomplete dechlorination in time-intensive tests. In these studies, ethanol was added alone, with FYE, or with a surrogate FYE (SFYE, a blend of VFAs expected to contribute reducing equivalents comparable to FYE, but without FYE's micronutrient contribution). Results are shown in Figure 8. All pertinent constituents are shown on the graph for each test performed.

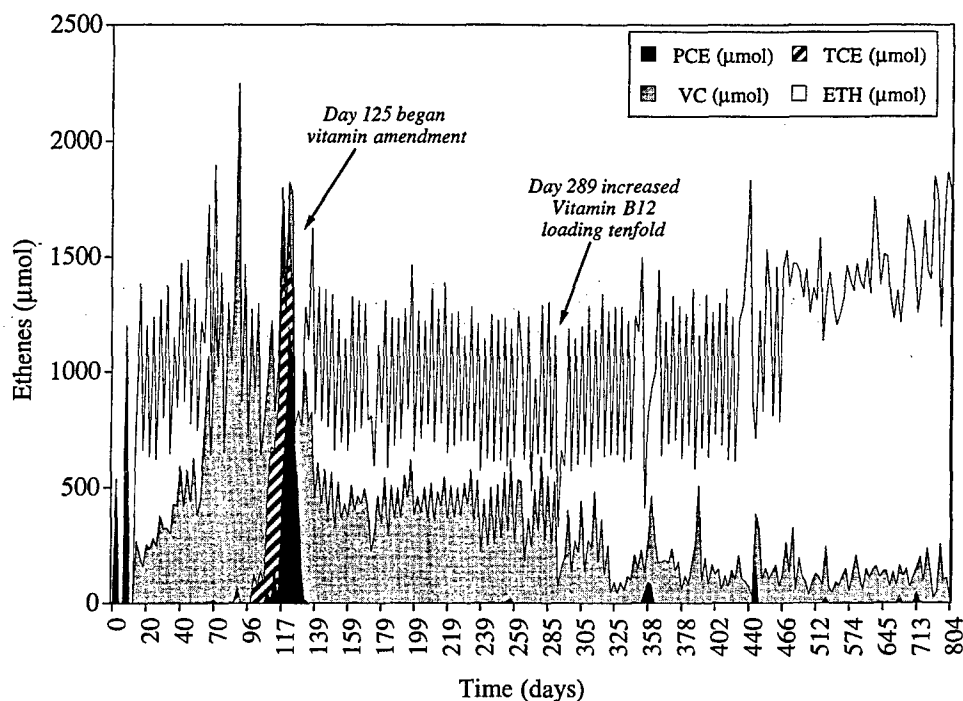


FIGURE 9. Results of PCE dechlorination in a butyric acid-amended dechlorinating culture at a 2:1 donor:PCE ratio over 2.2 yr of operation.

In each case, the H_2 peaked at approximately 4000 nmol ($10^{-2.8}$ atm), but only the lower portion of the H_2 curve is shown. The VFAs butyric, propionic, isobutyric, isovaleric, and hexanoic acids were quantified during these tests; however, only butyric and propionic acids were present in significant amounts.

When ethanol alone was added (Figure 8a), we observed the typical response—dechlorination and methanogenesis initially proceeded very rapidly, then methanogenesis ceased and dechlorination continued at a much slower rate as H_2 levels fell below the level that supported methanogenesis. Note that propionic acid—a potential electron donor—was present in the culture, apparently produced during the degradation of ethanol. Its slow degradation after the H_2 levels fell below 20 nmol ($10^{-5.1}$ atm) continued to fuel dechlorination, though a sharp decrease in rate was observed.

Addition of FYE with the ethanol (Figure 8b) resulted in a less sharply delineated “break” in the rate of PCE dechlorination after the H_2 produced from ethanol degradation was depleted. The primary factor in this case was the presence of butyric acid (a constituent of FYE). Butyric acid was not degraded while H_2 was present at high levels, but when H_2 fell below about 30 nmol ($10^{-4.9}$ atm), butyric acid was degraded readily. Addition of SFYE along with ethanol (Figure 8c) yielded results that were very similar to those of ethanol plus FYE—confirming our suspicion that FYE was serving as an important supplemental electron donor as well as nutritional supplement during the long-term tests.

Butyric Acid Enrichment Culture. We wished to demonstrate, over a period of years, that one of the low- H_2 electron donors could serve as an effective, stable stimulator of dechlorination even in the presence of competing methanogens. We have operated the high-PCE methanol enrichment culture (31) and H_2 -amended purified cultures of the dechlorinator (7) over long periods with the retention of dechlorination. However, these cultures essentially operate in the absence of methanogens—through inhibition by high PCE in the case of the methanol cultures and through purification in the case of the H_2 cultures. Thus, it was important to document dechlorination stability under conditions of a potentially functioning methanogenic population. The dechlorination product formation in the butyric acid enrichment culture is shown in Figure 9. After nutritional

problems were overcome after day 100 by the addition of vitamin solution containing vitamin B_{12} (7, 30), this culture has dechlorinated about 83% of the added PCE to ETH and the remainder to VC in a very stable and continuous manner.

Discussion

During the long-term electron donor comparison experiments, we expected to observe significant differences in the partitioning of reduction equivalents to PCE dechlorination and methanogenesis when we used different electron donors. We expected that cultures fed lactic acid or ethanol, which are degraded more rapidly and produce higher levels of H_2 , would eventually fail as methanogens came to predominate the culture and dechlorinators were marginalized. Conversely, we believed that cultures fed the more slowly fermentable, low H_2 -producing donors, butyric and propionic acids, would result in low levels of H_2 that would be less available to methanogens and would result in a predominance of dechlorination. However, during long-term operation, we observed nearly equally good establishment and maintenance of dechlorination, regardless of the electron donor fed. The only difference noted was the significantly slower methanogenic activity development in the propionic acid-fed cultures (prior to the onset of acetotrophic activity). While the propionic acid enrichment did seem to exclude a vigorous methanogenic population from startup, it did not then concurrently result in significantly better dechlorination than enrichments using other electron donors.

The expected differences among electron donors were observed during short-term, time-intensive tests. The slowly degraded, low- H_2 -producing substrates (butyric and propionic acids) did support dechlorinators while minimizing, and in the case of propionic acid essentially excluding, methanogenic competition. At a 1:1 donor:PCE ratio, lactic acid degradation also produced a much lower H_2 peak than ethanol and resulted in less competing methanogenesis. In contrast, the degradation of ethanol, both at 1:1 and 2:1 ratios and lactic acid at a 2:1 ratio resulted in orders of magnitude higher H_2 that fueled initial rapid dechlorination and methanogenesis; however, as the donor and H_2 were depleted, dechlorination slowed drastically, often leaving significant quantities of PCE that were then only slowly degraded.

In their studies of anaerobic aquifer sediments from a Traverse City, MI, site that were amended with fatty acid mixtures, Gibson et al. (26) observed that lactic acid was quickly depleted and probably did not persist long enough to support dechlorination, whereas butyric acid persisted for a longer period of time and was a better amendment for stimulating dechlorination. Propionic acid degradation was not observed in their microcosms amended with the fatty acid mixtures—perhaps, they suggest, because of inhibition by high levels of H_2 and/or acetic acid. In microcosms amended with propionic acid alone, dechlorination was supported after a lag period (25). The stability of our butyric acid-amended enrichment culture and the high ETH production that it exhibited at non-inhibitory PCE concentrations in the presence of a vigorous methanogenic community are encouraging. This information demonstrates the ability of dechlorinators to maintain their important role in a community containing other hydrogenotrophs.

We suspected and then confirmed through additional testing that the addition of FYE (an unfortunately required micronutrient supplement) significantly influenced the outcome of the long-term tests. The time-intensive studies of ethanol with added supplements clearly showed the importance of FYE-contributed VFAs—notably butyric acid—in providing slowly released H_2 to fuel the continued dechlorination of PCE that remained after ethanol was depleted. While we know that nutrients contained in yeast extract are required for the growth of pure cultures of the dechlorinating organism (7) and for the high-PCE methanol-fed source culture (31), we have not yet determined whether this nutritional requirement holds for cultures amended with the electron donors tested in this study. It is possible that the complex mixed community itself would provide the missing nutrients.

In addition to the influence of added FYE on donor performance, the pathway of fermentation of the donor will also greatly determine whether it is suitable or not. We observed propionic acid accumulation in both lactic acid- and ethanol-fed cultures. We have also observed propionic acid accumulation in microcosms amended with these donors (32). These pathways are well documented. Lactic acid can be degraded to propionic acid by *Veillonella* (33), *Propionibacterium*, *Megasphaera*, *Selenomonas*, and various *Clostridia* (34). Ethanol fermentation to propionic acid, a more recent discovery, was first reported (35) for *Clostridium neopropionicum* (36) and is also carried out by *Desulfobus propionicus* (37) and *Pelobacter propionicus* (38). This pathway has been shown to be important in some natural environments (39). Other possibilities exist; for example, butyric acid formation from ethanol and acetate has been documented for *Clostridium kluyveri* (34). The accumulated propionic acid (in addition to FYE) in our cultures contributed to the pool of slowly degradable donor and undoubtedly facilitated continued dechlorination after the primary donors were depleted.

Thus, the fermentation pathways of the donors tested did not follow the simple model of degradation to acetic acid and H_2 . A much more complicated scenario was observed. In some ecosystems, adding ethanol or lactic acid may be almost the equivalent of adding propionic acid—a slow-release H_2 donor. Cost and ease of handling could then help determine which type of addition is more practical. This study has shown that fate of electron donors and their fermentation products—including not only H_2 but also other intermediates as well—is of critical importance for understanding dechlorinating communities. Site-by-site determinations, not only of dechlorinating capacity but also of donor fate will be useful for evaluating treatment schemes for bioremediation and will also be of great value in understanding results at naturally attenuated sites.

Acknowledgments

This research was supported by the U.S. Air Force Armstrong Laboratory, Environmental Quality Directorate, Tyndall AFB, FL, under Contract F08635-93-C-0077, and through a Selected Professions Fellowship from the American Association of University Women Educational Foundation.

Literature Cited

- (1) Deckard, L. A.; Willis, J. C.; Rivers, D. B. *Biotechnol. Lett.* 1994, 16, 1221–1224.
- (2) Fathepure, B. Z.; Nengu, J. P.; Boyd, S. A. *Appl. Environ. Microbiol.* 1987, 53, 2671–2674.
- (3) Fathepure, B. Z.; Boyd, S. A. *FEMS Microbiol. Lett.* 1988, 49, 149–156.
- (4) Fathepure, B. Z.; Boyd, S. A. *Appl. Environ. Microbiol.* 1988, 54, 2976–2980.
- (5) Mohn, W. W.; Tiedje, J. M. *Microbiol. Rev.* 1992, 56, 482–507.
- (6) Terzenbach, D. P.; Blaut, M. *FEMS Microbiol. Lett.* 1994, 123, 213–218.
- (7) Maymó-Gatell, X.; Tandoi, V.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* 1995, 61, 3928–3933.
- (8) Holliger, C.; Schraa, G.; Stams, A. J. M.; Zehnder, A. J. B. *Appl. Environ. Microbiol.* 1993, 59, 2991–2997.
- (9) Holliger, C.; Schumacher, W. *Antonie van Leeuwenhoek* 1994, 66, 239–246.
- (10) Scholz-Muramatsu, H.; Szewzyk, R.; Szewzyk, U.; Gaiser, S. *FEMS Microbiol. Lett.* 1990, 66, 81–86.
- (11) Neuman, A.; Scholtz-Muramatsu, H.; Diekert, G. *Arch. Microbiol.* 1994, 162, 295–301.
- (12) Scholz-Muramatsu, H.; Neuman, A.; Messmer, M.; Moore, E.; Diekert, G. *Arch. Microbiol.* 1995, 163, 48–56.
- (13) Krumholz, L. R. A new anaerobe that grows with tetrachloroethylene as an electron acceptor. *Abstracts of the 95th General Meeting of the American Society for Microbiology*; American Society for Microbiology: Washington, DC, 1995; Paper Q-24, p 403.
- (14) Sharma, P.; McCarty, P. L. *Appl. Environ. Microbiol.* 1996, 62, 761–765.
- (15) Gerritse, J.; Renard, V.; Pedro Gomes, T. M.; Lawson, P. A.; Collins, M. D.; Gottschal, J. C. *Arch. Microbiol.* 1996, 165, 132–140.
- (16) Stover, M. A. M.S. Thesis, Cornell University, Ithaca, NY, 1993.
- (17) Smatlak, C. R.; Gossett, J. M.; Zinder, S. H. *Environ. Sci. Technol.* 1996, 30, 2850–2858.
- (18) McNerney, M. J.; Bryant, M. P.; Hespell, R. B.; Costerton, J. W. *Appl. Environ. Microbiol.* 1981, 41, 1029–1039.
- (19) Boone, D. R.; Bryant, M. P. *Appl. Environ. Microbiol.* 1980, 40, 626–632.
- (20) Stieb, M.; Schink, B. *Arch. Microbiol.* 1985, 140, 387–390.
- (21) Schink, B.; Friedrich, M. *FEMS Microbiol. Rev.* 1994, 15, 85–94.
- (22) Thauer, R. K.; Jungermann, K.; Decker, K. *Bacteriol. Rev.* 1977, 41, 100–180.
- (23) Major, D. W.; Hodgins, E. W.; Butler, B. J. In *On-Site Bioreclamation—Processes for Xenobiotic and Hydrocarbon Treatment*; Hincsee, R. E., Olfenbittel, R. F., Eds.; Butterworth-Heinemann: Boston, MA, 1991; pp 113–133.
- (24) Pavlostathis, S. G.; Zhuang, P. *Chemosphere* 1993, 27, 585–595.
- (25) Gibson, S. A.; Sewell, G. W. *Appl. Environ. Microbiol.* 1992, 58, 1392–1393.
- (26) Gibson, S. A.; Roberson, D. S.; Russell, H. H.; Sewell, G. W. *Environ. Toxicol. Chem.* 1994, 13, 453–460.
- (27) Beeman, R. E.; Howell, J. E.; Shoemaker, S. H.; Salazar, E. A.; Buttram, J. R. In *Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds*; Hincsee, R. E., Leeson, A., Semprini, L., Ong, S. K., Eds.; Lewis Publishers: Boca Raton, FL, 1993; pp 14–27.
- (28) Beeman, R. E.; Shoemaker, S. H.; Buttram, J. R. In situ demonstration of anaerobic bioremediation of chlorinated ethenes. Presented at the AICHE 1993 Summer National Meeting, August 18, 1993, Seattle, WA.
- (29) Freedman, D. L.; Gossett, J. M. *Appl. Environ. Microbiol.* 1989, 55, 2144–2151.
- (30) Fennell, D. E.; Stover, M. A.; Zinder, S. H.; Gossett, J. M. In *Bioremediation of Chlorinated Solvents*; Hincsee, R. E., Leeson, A., Semprini, L., Eds.; Battelle Press: Columbus, OH, 1995; pp 9–16.
- (31) DiStefano, T. D.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* 1992, 58, 3622–3629.
- (32) Smatlak, C. R.; Gossett, J. M. Cornell University, unpublished results, 1996.
- (33) Delwiche, E. A.; Pestka, J. J.; Tortorello, M. L. *Annu. Rev. Microbiol.* 1985, 39, 175–193.

- (34) Gottschalk, G. *Bacterial Metabolism*; Springer-Verlag: New York, 1986.
- (35) Samain, E.; Albagnac, G.; Dubourguier, H. C.; Touzel, J. P. *FEMS Microbiol. Lett.* **1982**, *15*, 69-74.
- (36) Tholozan, J. L.; Touzel, J. P.; Samain, E.; Grivet, J. P.; Prensier, G.; Albagnac, G. *Arch. Microbiol.* **1992**, *157*, 249-257.
- (37) Laanbroek, H. J.; Abee, T.; Voogd, I. L. *Arch. Microbiol.* **1982**, *133*, 178-184.
- (38) Schink, B. *Arch. Microbiol.* **1984**, *137*, 33-41.

- (39) Schink, B.; Phelps, T. J.; Eichler, B.; Zeikus, J. G. *J. Gen. Microbiol.* **1985**, *131*, 651-660.

*Received for review September 9, 1996. Revised manuscript received November 8, 1996. Accepted November 12, 1996.**
ES960756R

* Abstract published in *Advance ACS Abstracts*, February 1, 1997.

Comparison of Alternative Electron Donors to Sustain PCE Anaerobic Reductive Dechlorination

*Donna E. Fennell, Michael A. Stover,
Stephen H. Zinder, and James M. Gossett*

ABSTRACT

Anaerobic reductive dechlorination of tetrachloroethene (PCE) to ethene (ETH) appears to use hydrogen as the direct electron donor (DiStefano et al. 1992). Hydrogen addition may be problematic for large-scale treatment systems. Adding an electron donor which is fermented to hydrogen may be more practical. Competition for substrate or reduction equivalents by methanogens should be minimized. Studies were performed with methanol, ethanol, lactic acid, and butyric acid to determine their suitability for maintaining reductive dechlorination by an anaerobic mixed culture. Electron donors were examined in semicontinuously operated serum bottles with a nominal PCE concentration of $110 \mu\text{mol/L}$ (neglecting partitioning to the gas space) and a 2:1 ratio of electron donor to PCE on an equivalent basis. The patterns of electron donor degradation, hydrogen formation, dechlorination, and methanogenesis were determined for each substrate. Dechlorination was sustained better with butyric acid, lactic acid, or ethanol than with methanol. Amendment with methanol stimulated methanogenesis and resulted in less complete dechlorination. Amendment with lactic acid or ethanol resulted in a hydrogen peak of 10^{-3} atm in 2 to 3 h. Butyric acid gave the most promising results. It was degraded about 10 times more slowly than ethanol or lactic acid, and its degradation constantly maintained 10^{-4} atm of hydrogen during the test. The data suggest that if hydrogen is supplied at low concentrations and low rates, dechlorination is favored over methanogenesis.

INTRODUCTION

Microbially mediated anaerobic reductive dechlorination of the common groundwater contaminants PCE and trichloroethene (TCE) has been investigated as a potential remediation tool since the early 1980s (Bouwer and McCarty 1982, 1983, 1985; Bouwer et al. 1981; Parsons et al. 1985; Vogel et al.

1987; Vogel and McCarty 1985). PCE and TCE are reductively dechlorinated to dichloroethene isomers (DCEs), vinyl chloride (VC), and ETH (Freedman and Gossett 1989), and in some cultures ethene is converted to ethane (Holliger et al. 1993). Many substrates such as glucose, sucrose, acetate, lactic acid, and methanol have been shown to support dechlorination both in microbial cultures and in sediment microcosms (Bouwer and McCarty 1983; Freedman and Gossett 1989; Gibson and Sewell 1992; Holliger et al. 1993). The dechlorinating organism(s) present in the mixed culture used during this study apparently use(s) hydrogen, which is evolved during degradation or conversion of more complex substrates, to carry out dechlorination (DiStefano et al. 1992). The dechlorinating organisms are not methanogens, but compete with hydrogenotrophic methanogens for available hydrogen. Attempts to sustain the dechlorinating mixed culture on hydrogen alone have been unsuccessful thus far without the addition of a complex nutrient source such as sludge supernatant (DiStefano et al. 1992; Tandoi et al. 1994). When PCE concentrations that were inhibitory to methanogens were used, methanol was an effective electron donor for dechlorination (DiStefano et al. 1992). The methanol was converted to acetate in these cultures, and the dechlorinating organisms presumably scavenged hydrogen from acetogens. When methanogenesis was not inhibited, competition for methanol or reducing equivalents by methanogens was a very important sink for electron donor.

The objective of this study was to compare the suitability of the electron donors lactic acid, ethanol, butyric acid, and methanol for maintaining dechlorination in a mixed anaerobic culture at noninhibitory PCE concentrations. For each substrate, the completeness of dechlorination during long-term operation and the patterns of hydrogen production, dechlorination, and methanogenesis during short-term tests were examined in semicontinuously operated serum bottles. The results were examined to determine if the pattern of hydrogen production influenced the favorability of dechlorination over methanogenesis.

EXPERIMENTAL PROCEDURES

Microbial Culture

The source culture used during these experiments was enriched with methanol, PCE, and yeast extract, operated with a nominal hydraulic retention time (HRT) of 40 days at 35°C and is described in detail elsewhere (DiStefano et al. 1992). For these experiments, aliquots of 100 mL were removed from the source culture and placed in 160-mL serum bottles. The bottles were capped with gray-butyl, Teflon™-lined septa (Wheaton) and crimped with aluminum caps. Following anaerobic transfer of the culture to the serum bottles, the bottles were maintained with the source culture protocol (addition of 55 µmol PCE, 156 µmol methanol, and 2 mg of prefermented yeast extract on Day -4 and Day -2) for 4 days to ensure that the transfer was successful. After 4 days the experiment was begun (defined as Day 0).

Cultures were grown in a basal salts medium, which has been used to develop and work with this culture. The solution was adapted (Freedman and Gossett 1989) from one used for culturing methanogens (Zeikus 1977). Prefermented yeast extract consisted of an anoxic mixture of 5 g yeast extract and 90 mL distilled water to which 10 mL of the source culture was added. The mixture was allowed to ferment for 10 days prior to use to remove readily available electron donor. At each feeding, 50 μ L of this solution was added as a source of trace nutrients.

Experimental Protocol

During the electron donor experiment, duplicate bottles were amended every second day with 11 μ mol PCE and either methanol (29.3 μ mol), ethanol (44 μ mol), lactic acid (44 μ mol), butyric acid (44 μ mol), or no electron donor. The electron donor was added at a 2:1 ratio to the PCE fed on an equivalent basis (176 μ eq electron donor to 88 μ eq PCE). Control bottles received PCE but no electron donor. All bottles were fed prefermented yeast extract as a trace-nutrient source.

On Day 0 and on every second day thereafter during long-term operation, the headspace of each bottle was sampled for dechlorination products, hydrogen, and methane, and then feeds were added. Every fourth day, after headspace samples were removed, an anoxic purge and a 10-mL exchange of culture and basal medium were made which resulted in a nominal HRT of 40 days. When liquid was removed, samples were taken for measurement of pH (and fatty acids for some bottles). After the basal medium exchange and anoxic purge, the bottles were fed.

The bottle sets were maintained with their respective protocols for 42 to 52 days for a long-term determination of the suitability of the various substrates as electron donors for PCE dechlorination. Time-intensive studies were performed initially within the first three weeks of set-up and finally during the last few days of operation on the same bottles to give a more detailed picture of culture performance, during which the disappearance of electron donor and the formation of dechlorination products, hydrogen, and methane were followed.

To initiate the time-intensive studies, the bottles were purged, 10 mL of culture was removed, and 9.0 mL of a PCE-saturated basal medium (1,200 μ M PCE) and 1 mL of regular basal medium were added. Methanol and butyric acid were added to the bottles in neat form, whereas lactate and ethanol were added as 20% stock solutions in distilled water. Prefermented yeast extract was not added during the time-intensive studies to allow more accurate determination of the fate of reduction equivalents. Headspaces were analyzed periodically for 48 h and six to eight 0.25-mL liquid samples were removed during the test to monitor electron donor degradation.

Analytical Methods

A gas chromatography system incorporating packed columns, two flame-ionization detectors, a reduction-gas detector, a hot-wire detector, and

switching valves to divert streams of carrier gas to specific detectors was used to quantify PCE, TCE, VC, ETH, CH_4 , and H_2 in 0.1 mL gas samples. The method has been described elsewhere (Freedman and Gossett 1989).

A Perkin-Elmer Corporation Model 8500 gas chromatograph with a 0.53-mm Nukol® capillary column and flame ionization detector was used for analysis of butyric acid. The nitrogen carrier gas flowrate was 10 mL/min (80 PSI), and the flame was maintained with hydrogen (40 PSI, 40 mL/min) and air (40 PSI, 450 mL/min). The injector temperature was 200°C and the detector temperature was 250°C. The column was held at 110°C for 4 min then ramped to 150°C at 15°C per min and held for 30 s. Culture samples of 0.25 or 0.5 mL were filtered and preserved to pH 1 to 2 with 5 or 10 μL of 6 N H_2SO_4 . Then 6N NaOH was added to bring the sample pH to 4 prior to analysis.

Lactic acid was determined with a Hewlett Packard 1090 high-performance liquid chromatograph (HPLC) with a 300-mm HPX-87H ion-exchange column (Bio-Rad Laboratories). The mobile phase was 0.013N H_2SO_4 , and the temperature was 65°C. Culture samples of 0.25 or 0.5 mL were filtered and preserved with 5 or 10 μL of 6N H_2SO_4 .

Ethanol and methanol were determined by employing an enzymatic/spectrophotometric method (Herzberg and Rogerson 1985). Samples were prepared in the same way as for HPLC.

RESULTS

Long-Term Operation

Initially in all of the bottles, most of the added PCE was converted to ETH. As the test progressed, dechlorination became less complete to varying degrees in all bottles. The decline was most pronounced in the methanol-amended bottles. In these methanol-amended bottles near the end of the study, at the end of a 2-day period, up to 20% of the PCE added remained and 70 to 80% of the reduction equivalents added as methanol were channeled to methanogenesis (Figure 1). In contrast, at the end of a 2-day period in a butyric-acid-amended bottle, PCE was completely absent, having been largely converted to ETH with only 10 to 35% remaining as VC. Of the reduction equivalents added as butyric acid, 42% was used for dechlorination, while the remainder was used for methane production. Ethanol, lactic acid, and butyric acid all sustained more complete dechlorination than methanol; however, all of the bottles exhibited some loss of dechlorination efficiency over time (data not shown). It was later shown with butyric-acid-amended cultures that loss of efficiency and eventual failure after 70 days under the same operational conditions as are described here were a result of nutrient limitation.

Final Time-Intensive Test

Time-intensive tests revealed more detail about how the electron donors were converted to hydrogen during 48-h periods following feeding. Final time-intensive tests were considered closer to "steady conditions" and will be

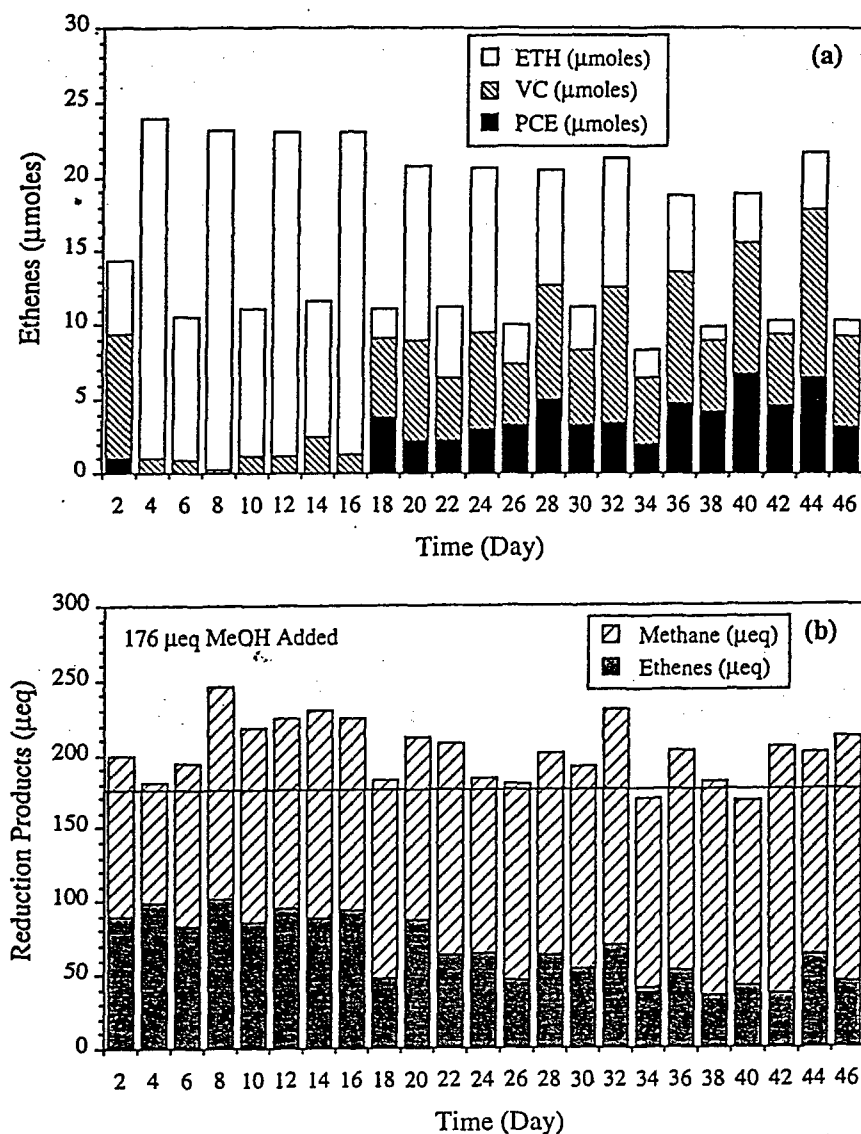


FIGURE 1. Results of dechlorination and reduction product distribution during long-term operation of a methanol-amended culture.

reported here. Graphs of dechlorination, methane production, and hydrogen levels during the final time-intensive test for an ethanol-amended bottle are shown in Figure 2. During the final time-intensive test, ethanol-amended bottles produced a pool of 2,500 and 3,000 nmol of hydrogen (10^{-3} atm) after

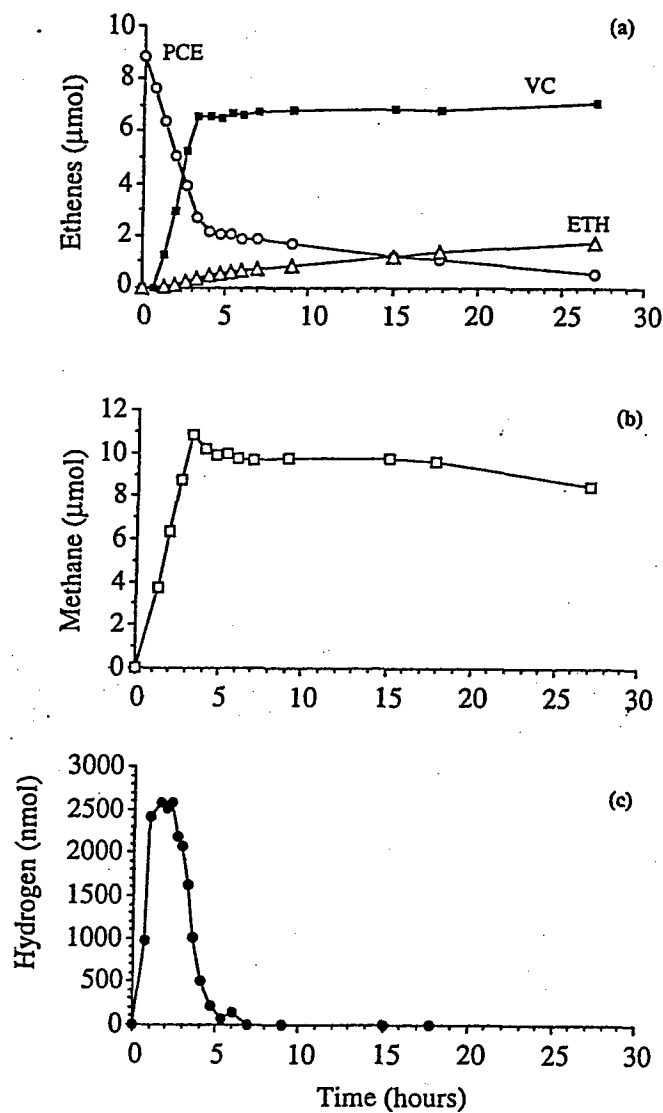


FIGURE 2. Results of dechlorination, methanogenesis, and hydrogen production during the final time-intensive study in an ethanol-amended bottle.

only 2 h and hydrogen was then quickly used primarily for methanogenesis. Dechlorination continued slowly in the ethanol-amended bottles, but PCE remained after 27 h. A similar pattern of hydrogen formation was observed in lactic-acid-amended bottles; however, PCE dechlorination was even less com-

plete than in the ethanol-amended bottles. The butyric-acid-amended bottles, in contrast, exhibited no hydrogen peak, only a steady pool of 200 to 250 nmol per bottle (10^{-4} atm) over a 48-h period (data not shown). PCE was completely converted to VC and ETH after 15 h in the butyric-acid-amended bottles. Addition of methanol, which is not converted directly to hydrogen, resulted in a hydrogen pool of less than 100 nmol per bottle (10^{-4} atm) during the 48 h after feeding. However, most of the reduction equivalents added were channeled directly to methanogenesis by methanol-using methanogens, and dechlorination of PCE was far from complete.

DISCUSSION

The source culture normally received PCE and electron-donor loadings that were five times those used in the experiment reported here. The results from this experiment, therefore, cannot be said to come from steady conditions since the bottles were only operated for 42 to 52 days—only about one HRT. However, the experiment can be used to compare the outcome of dechlorination when different electron donors are used.

The decline in the completeness of dechlorination in all of the bottles may have been, in part, a result of a population shift following the change in electron donor type and loading; however, it may also have been a result of nutrient limitation. A nutrient limitation was observed later in butyric-acid-enriched cultures, which were operated with the same protocol as the butyric-acid-amended bottles reported here. This nutrient limitation was overcome by adding a vitamin solution (Balch et al. 1979).

Because the methanogens were inhibited by the higher PCE loading rate in the source culture, but were free to grow in the bottles that received a lower PCE loading, they began to compete for hydrogen (or for the supplied substrate itself, in the case of the methanol-amended bottles). This explains the shift of reduction equivalents from dechlorination to methanogenesis during long-term operation. Thus, the nonmethanogenic substrates maintained dechlorination better than methanol. The electron donor that resulted in a lower hydrogen partial pressure, butyric acid, sustained dechlorination the best. More of the reducing equivalents released from its degradation continued to be channeled to dechlorination. The results suggest that non-methanogenic substrates that produce a low and steady supply of hydrogen will result in more complete, and better sustained, dechlorination of PCE and lesser chlorinated ethenes than those that produce a higher amount of hydrogen in a short amount of time. This information suggests that the dechlorinating organisms and hydrogenotrophic methanogens have different affinities for hydrogen.

REFERENCES

- Balch, W.E., G.E. Fox, L.J. Magrum, C.R. Woese, and R.S. Wolfe. 1979. "Methanogens: Reevaluation of a unique biological group." *Microbiology Reviews* 43: 260-296.

- Bouwer, E. J., and P. L. McCarty. 1982. "Removal of trace chlorinated organic compounds by activated carbon and fixed-film bacteria." *Environmental Science and Technology* 16 (12): 836-843.
- Bouwer, E. J., and P. L. McCarty. 1983. "Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions." *Applied and Environmental Microbiology* 45 (4): 1286-1294.
- Bouwer, E. J., and P. L. McCarty. 1985. "Utilization rates of trace halogenated organic compounds in acetate-grown biofilms." *Biotechnology and Bioengineering* XXVII 1564-1571.
- Bouwer, E. J., B. E. Rittmann, and P. L. McCarty. 1981. "Anaerobic degradation of halogenated 1- and 2-carbon organic compounds." *Environmental Science & Technology* 15 (5): 596-599.
- DiStefano, T. D., J. M. Gossett, and S. H. Zinder. 1992. "Hydrogen as an electron donor for the dechlorination of tetrachloroethene by an anaerobic mixed culture." *Applied and Environmental Microbiology* 58: 3622-3629.
- Freedman, D. L., and J. M. Gossett. 1989. "Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions." *Applied and Environmental Microbiology* 55 (9): 2144-2151.
- Gibson, S. A., and G. W. Sewell. 1992. "Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols." *Applied and Environmental Microbiology* 58 (4): 1392-1393.
- Herzberg, G. R., and M. Rogerson. 1985. "Use of alcohol oxidase to measure the methanol produced during the hydrolysis of D- and L-methyl-3-hydroxybutyric acid." *Analytical Biochemistry* 149: 354-357.
- Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder. 1993. "A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth." *Applied and Environmental Microbiology* 59 (6): 2991-2997.
- Parsons, F., G. B. Lage, and R. Rice. 1985. "Biotransformation of chlorinated organic solvents in static microcosms." *Environmental Toxicology and Chemistry* 4: 739-742.
- Tandoi, V., T. D. DiStefano, P. A. Bowser, J. M. Gossett, and S. H. Zinder. 1994. "Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture." *Environmental Science and Technology* 28 (5): 973-979.
- Vogel, T. M., C. S. Criddle, and P. L. McCarty. 1987. "Transformations of halogenated aliphatic compounds." *Environmental Science and Technology* 21 (8): 722-736.
- Vogel, T. M., and P. L. McCarty. 1985. "Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions." *Applied and Environmental Microbiology* 49 (5): 1080-1083.
- Zeikus, J. G. 1977. "The biology of methanogenic bacteria." *Bacteriology Reviews* 41 (2): 514-541.

Characterization of an H₂-Utilizing Enrichment Culture That Reductively Dechlorinates Tetrachloroethene to Vinyl Chloride and Ethene in the Absence of Methanogenesis and Acetogenesis

XAVIER MAYMÓ-GATELL,¹ VALTER TANDOI,^{1†} JAMES M. GOSSETT,²
AND STEPHEN H. ZINDER^{1*}

*Section of Microbiology¹ and School of Civil and Environmental Engineering,²
Cornell University, Ithaca, New York 14853-8101*

Received 23 February 1995/Accepted 5 September 1995

We have been studying an anaerobic enrichment culture which, by using methanol as an electron donor, dechlorinates tetrachloroethene (PCE) to vinyl chloride and ethene. Our previous results indicated that H₂ was the direct electron donor for reductive dechlorination of PCE by the methanol-PCE culture. Most-probable-number counts performed on this culture indicated low numbers ($\leq 10^4$ /ml) of methanogens and PCE dechlorinators using methanol and high numbers ($\geq 10^6$ /ml) of sulfidogens, methanol-utilizing acetogens, fermentative heterotrophs, and PCE dechlorinators using H₂. An anaerobic H₂-PCE enrichment culture was derived from a 10^{-6} dilution of the methanol-PCE culture. This H₂-PCE culture used PCE at increasing rates over time when transferred to fresh medium and could be transferred indefinitely with H₂ as the electron donor for the PCE dechlorination, indicating that H₂-PCE can serve as an electron donor-acceptor pair for energy conservation and growth. Sustained PCE dechlorination by this culture was supported by supplementation with 0.05 mg of vitamin B₁₂ per liter, 25% (vol/vol) anaerobic digester sludge supernatant, and 2 mM acetate, which presumably served as a carbon source. Neither methanol nor acetate could serve as an electron donor for dechlorination by the H₂-PCE culture, and it did not produce CH₄ or acetate from H₂-CO₂ or methanol, indicating the absence of methanogenic and acetogenic bacteria. Microscopic observations of the purified H₂-PCE culture showed only two major morphotypes: irregular cocci and small rods.

Tetrachloroethene (perchloroethylene or PCE) and trichloroethene (TCE) are commonly used organic solvents which have been released into the environment and have become major groundwater pollutants. PCE appears to be completely resistant to metabolism by aerobes, while TCE can be co-oxidized by certain nonspecific oxygenases (9). However, considerable evidence has accrued in studies of anaerobic microcosms and cultures for reductive dechlorination of PCE to TCE (10), dichloroethene (DCE) isomers (1, 14, 16), or vinyl chloride (VC) (12, 24). More importantly, complete dechlorination to ethene (ETH) (7, 12) or ethane (4) has been reported. It is not clear why some anaerobic systems only partially dechlorinate PCE while others effect complete dechlorination. Little is known about the identity of organisms responsible for reductive dechlorination in these systems, although the recent descriptions of strain PER-K23 (16) and *Dehalospirillum multivorans* (20), which are capable of reduction of PCE to *cis*-DCE, provide examples of what is likely to be a diversity of organisms capable of reductive dechlorination of chloroethenes.

We have been studying an anaerobic enrichment culture which uses methanol as the electron donor for reductive dechlorination of PCE to ETH. In initial studies (12), PCE was fed every 2 days at a dose of 3.5 μ mol/liter of culture medium. VC was the primary product, and most of the 0.32 mmol of methanol per liter that was added as an electron donor was used for methanogenesis. In a subsequent study (7), the PCE

and methanol doses were gradually increased until they reached 0.55 mmol of PCE per liter and 1.6 mmol of methanol per liter. This increase drastically improved the performance of the culture, with nearly complete conversion of PCE to ETH occurring within 4 days. The culture also had essentially ceased producing methane from methanol and, instead, the reducing equivalents from methanol which were not used for PCE reduction were used for acetogenesis.

More recent time course studies (22) have shown that the methanol-PCE culture was capable of reductive dechlorination of all chloroethenes. PCE was initially degraded quantitatively to VC. VC dechlorination to ETH did not usually commence until other chloroethenes were absent and VC dechlorination showed first-order kinetics. The culture also made small amounts of methane, usually after PCE depletion.

While methanol could serve as an electron donor for the culture and appeared to be especially well suited for sustained dechlorination, it was found in early studies that glucose, formate, and H₂ could also serve as electron donors (12). Studies with other cultures have shown that H₂ (16), fatty acids (14), and even toluene (21) could stimulate PCE dechlorination. We suspected that some of these donors were serving as sources of a more universal electron donor, H₂. We found that for the methanol-PCE culture, H₂ could readily replace methanol for several feedings (8), although eventually performance faltered. Good performance was restored by resuspension of the H₂-PCE culture in the supernatant from the methanol-PCE culture, suggesting that the latter contained growth factors required for dechlorination. The eubacterial cell wall synthesis inhibitor vancomycin inhibited PCE dechlorination and acetogenesis from methanol, but PCE dechlorination from H₂ was

* Corresponding author. Phone: (607) 255-2415. Electronic mail address: shz1@cornell.edu.

† Present address: CNR IRSA, 00198 Rome, Italy.

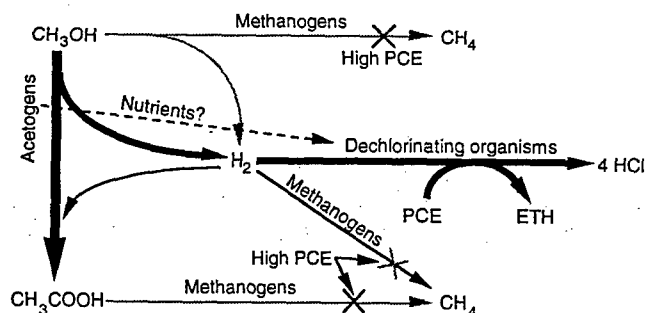


FIG. 1. Model, based on that of DiStefano et al. (8), for carbon and electron flow in a methanol-PCE anaerobic mixed culture.

not affected. This suggested that the PCE dechlorinator was resistant to vancomycin and that it could not use methanol directly but rather required methanol metabolism, most likely to provide H_2 or formate as the electron donor for reductive dechlorination.

Figure 1 shows a model of the metabolism of methanol and PCE by the dechlorinating culture which includes the hypotheses that H_2 is the actual electron donor for dechlorination and that a nutritional contribution is made to PCE dechlorinators by methanol-metabolizing organisms (6, 8). This model suggests that a better-defined culture on H_2 and PCE could be obtained if its nutritional needs were met. In this report, we examine the microbial populations in the methanol-PCE culture and describe the characterization of an H_2 -PCE culture derived from a 10^{-6} dilution of the methanol-PCE culture.

MATERIALS AND METHODS

Chemicals. High-performance liquid chromatography-grade PCE (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and methanol (Fisher Scientific, Springfield, N.J.) were used as culture substrates. H_2 was purchased from Empire Airgas, Inc., Elmira, N.Y. VC was obtained as a gas from Matheson Gas Products, Inc., Secaucus, N.J. Other chloroethenes were obtained from Aldrich Chemical Co. Ethane, ethene, and methane were purchased from Supelco, Bellefonte, Pa. All of the other chemicals used were reagent grade or better.

Analyses. For qualitative analysis of ethenes in most-probable-number (MPN) tubes, a model 1400 flame ionization detector-gas chromatograph (GC) (Varian, Walnut Grove, Calif.) was used with a stainless steel column (2 m by 3 mm) packed with 60/80 mesh Carbowax B-1% SP-1000 (Supelco) and operated isothermally at 200°C as described previously (22).

For quantitative analysis of chloroethenes and ETH, samples were analyzed with a model 8610 GC (SRI Instruments, Las Vegas, Nev.) equipped with a flame ionization detector and using the same column and operating conditions as described above. Peak areas were calculated by using the Peaksimple 2 software supplied with the GC and were compared to standard curves for chloroethenes (15). When we added 5 μ mol of PCE to a 27-ml crimp-top tube containing 10 ml of growth medium, the nominal concentration was 0.5 mmol/liter, while the estimated aqueous concentration (15) was 0.16 mmol/liter.

When methane and ETH were produced together, they were measured with a Varian 1400 GC using a Poropak R column as described previously (19). The detection limit for methane was ca. 0.1 Pa, or 1 nmol per tube. H_2 was analyzed by gas chromatography with an AGC 110 thermal conductivity GC (Hach Carle, Lehigh Valley, Pa.) using N_2 as the carrier gas as described previously (28), and the detection limit for H_2 was ca. 2 Pa. Headspace samples were 100 μ l in all cases. Acetate concentrations were quantified by high-performance liquid chromatography with a fast acid analysis column (Bio-Rad, Richmond, Calif.) and a solvent of 6.5 mM H_2SO_4 as previously described (31). Total organic carbon was measured with a model 5010 organic carbon analyzer (UIC Inc., Joliet, Ill.).

Growth medium and culture conditions. Methanol-PCE cultures were incubated in the growth medium and under conditions described previously (22). Unless stated otherwise, the basal medium contained the following (final concentration in grams per liter): NH_4Cl , 0.5; K_2HPO_4 , 0.4; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.05; resazurin, 0.001; and 10 ml of a trace metal solution (27) per liter modified by addition of 0.01 g of $NiCl_2 \cdot 6H_2O$ per liter. The medium was purged with N_2 and dispensed inside an anaerobic chamber into 27-ml crimp-top tubes (Bellco, Vineland, N.J.). The tubes were then sealed with aluminum-crimp Teflon-coated butyl rubber stoppers (Wheaton, Millville, N.J.) and autoclaved at 121°C for 55 min. The tube headspaces were flushed with sterile 70% N_2 -30%

CO_2 (Matheson Gas Products, Inc., Secaucus, N.J.) and received the following sterile and anaerobic additions before inoculation (final medium volume, \approx 10 ml per tube): $Na_2S \cdot 9H_2O$, 2 mM; $NaHCO_3$, 12 mM; filter-sterilized anaerobic digester sludge supernatant (SS), 25% (vol/vol); yeast extract (YE), 0.2 g/liter, or sodium acetate, 2 mM; vitamin solution (2) concentrated 10-fold, 0.5% (vol/vol).

The anaerobic sewage digester sludge for preparation of SS was obtained from the Ithaca Area Wastewater Treatment Facility. Several liters were collected at one time, to ensure uniformity. The sludge was initially clarified by centrifugation for 30 min at 8,000 rpm ($10,400 \times g$) with a Sorvall RC-2B centrifuge (DuPont-Sorvall, Wilmington, Del.). Samples were stored frozen at $-20^\circ C$ until use. Thawed samples were then centrifuged for 30 min at 18,000 rpm ($39,000 \times g$). The resulting liquid was neutralized to pH 7.0 with 1 M HCl and then prefiltered through a 0.8- μ m-pore-size Supor-800 membrane filter (Gelman Sciences, Ann Arbor, Mich.). The SS was then purged with N_2 and filter sterilized in an anaerobic chamber through a double 0.8-0.2- μ m-pore-size Acrodisc PF filter (Gelman) into sterile vials. In some instances, the SS was lyophilized in a Labconco LYPH-LOCK 4.5-liter Freeze Dry system, model 77510 (Labconco Corporation, Kansas City, Mo.). Preliminary tests showed that lyophilized SS performed as well as SS. The SS contained 75 mg of organic carbon per liter and \leq 0.05 mM acetate or other volatile fatty acids.

Unless otherwise stated, the inoculum size was 2% (vol/vol), all incubations were done in duplicate, and each experiment described was performed at least twice with similar results. Duplicate tubes performed very similarly in the experiments described (less than 5% difference in results for tubes under the same conditions), so the results are presented for individual tubes. Cultures that used H_2 as an electron donor were incubated upside down in the dark in an R76 incubator-shaker (New Brunswick Scientific, Edison, N.J.) operated at 35°C and 150 rpm. H_2 was added to the headspace as overpressure (0.67 atm, 67 kPa, or ca. 47.5 mmol/liter) immediately after inoculation. After several doses of PCE were consumed, $NaHCO_3$ was added to neutralize the HCl produced by the dechlorination process (22) and H_2 was added to replenish the headspace. The quantity of PCE added to a culture tube was estimated directly from the delivered syringe volume (7).

MPN determinations of the methanol-PCE culture. Three-tube MPN determinations were performed for a variety of microbial groups present in the methanol-PCE culture. All tubes contained the basal medium described above.

A master set of dilutions was made in 160-ml serum vials containing 45 ml of basal medium, which was used for all subsequent dilutions. H_2 - CO_2 -utilizing methanogens and acetogens were enumerated by using medium to which H_2 was added at 130 kPa (ca. 90 mmol/liter) after inoculation. Methanol-utilizing methanogens and acetogens were enumerated by using medium to which 24 mM methanol was added, while 40 mM Na acetate was used to culture acetate-utilizing methanogens. Sulfidogens (e.g., sulfate reducers) were enumerated by using medium which received 5 mM Na acetate, H_2 at 130 kPa, and 10 mM $Na_2S_2O_3$, which is used by more sulfidogens than sulfate (26). Fermentative heterotrophs were enumerated in medium to which an additional 1 g of YE per liter and 0.25 g each of glucose, cellobiose, xylose, and sucrose per liter were added. Tubes were considered positive when significantly greater turbidity was observed than in control dilutions lacking these additions and when the appropriate product (methane or acetate) was detected.

Methanol-utilizing PCE dechlorinators were enumerated in medium to which 12 mM methanol had been added together with the equivalent of 0.2 mmol of PCE per liter. Cultures were considered positive if they produced greater-than-trace quantities of reduction products (generally, VC and ETH were the only products detected). H_2 -utilizing PCE dechlorinators were enumerated and assayed in a similar manner, except that H_2 at 130 kPa replaced methanol as the electron donor.

Microscopy. A Zeiss Standard 18 microscope was used for phase-contrast observation of cells and for epifluorescence microscopy to detect organisms with the cofactor F_{420} autofluorescence typical of most methanogens as described previously (29). For photomicroscopy, cells were spread out on an agar-coated slide and stained with 0.01% acridine orange. The cells were observed with a Zeiss LSM-10 laser scanning microscope with either phase-contrast or epifluorescence (488 nm) imaging.

RESULTS

Viable counts of microbial populations in the methanol-PCE culture. We used the MPN technique to estimate the numbers of members of various microbial groups in the methanol-PCE culture (Table 1). Neither methanol- nor acetate-utilizing methanogens were detected in the culture. Relatively few H_2 - CO_2 -utilizing methanogens were detected, and microscopic examination of the highest positive dilution tubes showed spirals with the distinctive morphology of *Methanospirillum* sp., a methanogen capable of using either H_2 - CO_2 or formate (2). High numbers of methanol-utilizing acetogens were found. A preliminary characterization of these chain-forming cocci indicated that they stained gram positive, re-

TABLE 1. MPN determinations for various microbial populations in a methanol-PCE culture

Organism type	MPN (ml ⁻¹)	Time (wks) until highest dilution was positive	Predominant morphology in highest positive dilution
H ₂ -CO ₂ -utilizing methanogens	2.3 × 10 ⁴	5	Long spirals
Methanol-utilizing methanogens	≤2.3		
Acetate-utilizing methanogens	≤2.3		
H ₂ -CO ₂ -utilizing acetogens	≤2.3 × 10 ⁴		
Methanol-utilizing acetogens	9.3 × 10 ⁶	2	Cocci in chains and doublets
H ₂ -utilizing thiosulfate reducers	9.3 × 10 ⁵	2	Small spirals
Fermentative heterotrophs	2.3 × 10 ⁷	2	Various rods
Methanol-utilizing PCE dechlorinators	2.3 × 10 ³	5	
H ₂ -utilizing PCE dechlorinators	2.3 × 10 ⁶	5	Small and large rods, irregular cocci, some small spirals

quired SS for growth, could not use H₂-CO₂ for growth, and did not significantly dechlorinate PCE by using either methanol or H₂ as the electron donor (23). The numbers of H₂-CO₂-utilizing acetogens were lower than those of methanogens. There were relatively high counts of sulfidogens in the culture, and the predominant morphotype in the highest positive dilution tubes resembled desulfovibrios. The highest MPN counts were for fermentative heterotrophs capable of growing in medium containing YE and sugars.

In MPN dilution tubes receiving methanol and PCE, the highest dilution which showed products of reductive dechlorination was 10⁻³, in which VC was detected within 3 weeks. There was growth in the methanol-PCE tubes to a dilution of 10⁻⁷, and chain-forming cocci similar to those seen in tubes receiving methanol without PCE were found in these tubes.

In MPN dilution tubes receiving H₂ and PCE, serial dilutions became positive for VC production sequentially until, after 5 weeks of incubation, two of three 10⁻⁶ dilution tubes were positive for VC production, with the third tube becoming positive approximately 1 week later. Although analysis of PCE and its products was qualitative, VC was the only daughter product initially detected in positive tubes until PCE was depleted, at which time ETH was detected. Microscopic examination of these cultures revealed small and large rods, individual irregular cocci, and *Desulfovibrio*-like spirals. These 10⁻⁶ dilution tubes consumed several subsequent doses of PCE, and transfers from these cultures were the source of the purified H₂-PCE cultures described in the rest of this communication.

PCE utilization by the purified H₂-PCE culture. Figure 2 shows that after the H₂-PCE culture was transferred into fresh medium, the rate of PCE utilization increased over time, indicating growth of the PCE-dechlorinating organisms. The major dechlorination products detected were VC and ETH, which were produced nearly stoichiometrically from PCE (there was always some loss of chloroethenes into or through the stoppers). By day 6, the culture was able to consume the equivalent of 1 mmol of added PCE per liter of culture medium in 15 h. Table 2 shows that cultures not provided with H₂ did not dechlorinate PCE and that methanol did not serve as an electron donor for PCE dechlorination. In cultures provided with PCE, there was greater disappearance of H₂ than in cultures lacking PCE or an inoculum, and the net amount consumed, 4.7 mmol/liter, was approximately equal to the amount required to reduce 1.66 mmol of PCE per liter to VC (4.9 mmol/liter). Moreover, cultures provided with H₂ or methanol in the presence or absence of PCE produced neither methane (data not shown) nor acetate (Table 2), indicating the complete absence of methanogens and acetogens, while in dechlorinating cultures the initial acetate concentration decreased as PCE was being consumed.

Nutritional characterization of the H₂-PCE culture. The H₂-PCE culture could be transferred indefinitely in medium containing 0.2 g of YE per liter, 25% (vol/vol) SS, and a vitamin solution as nutritional supplements. It was desirable to replace YE with nutrients which would support less growth of contaminants while still supporting PCE dechlorination.

Our initial studies showed that deleting YE from the growth medium did not allow sustained PCE dechlorination (data not shown). Since it was possible that YE or some product derived from it was serving as a carbon source, we tested whether YE could be replaced with acetate, a common carbon source that is used by many anaerobes but is not utilizable as an energy source by most anaerobes. Figure 3 shows that acetate greatly stimulated dechlorination by the H₂-PCE culture when YE was not present. Formation of dechlorination products (almost completely VC and ETH) essentially stopped after day 5 in cultures grown without acetate, while they continued to increase rapidly when 2 mM Na acetate was present. Acetate concentrations higher than 2 mM were not more stimulatory (data not shown). Cultures could be transferred indefinitely with 2 mM Na acetate (replacing YE), 25% (vol/vol) SS, and vitamins. The MPN of YE-utilizing fermentative heterotrophs in this medium was 2.3 × 10⁶, a 10-fold reduction compared with cultures receiving YE, such as the methanol-PCE culture (Table 1).

TABLE 2. Effects of electron donors on PCE dechlorination (mainly to VC and ETH) and acetogenesis by a purified H₂-PCE culture

Addition	Product concn (mmol/liter) ^a on day 14	H ₂ decrease (%) ^b on day 14	Acetate concn (mM) ^c	
			Day 1	Day 14
H ₂ + PCE	1.66	10.1	1.97	1.60 ^d
Methanol + PCE	0	NA ^e	1.94	2.03
No electron donor + PCE	0	NA	2.04	2.06
No electron donor (no PCE)	NA	NA	1.97	1.98
H ₂ (no PCE)	NA	3.3	2.06	1.94
Methanol (no PCE)	NA	NA	1.97	1.97
H ₂ (no PCE, no inoculum)	NA	3.2	2.07	2.06

^a Values obtained from PCE dechlorination to almost completely ETH and VC.

^b Percentage obtained from the mean of duplicate tubes (sampling headspace). Standard deviations were 1.5 for the H₂-PCE cultures and 1.0 for the H₂-no PCE and H₂-no PCE-no inoculum cultures.

^c Mean concentration of duplicate tubes (sampling liquid phase). Standard deviations were 0.3 for the H₂-PCE cultures and 0 to 0.1 for the rest of the cultures.

^d Value measured after 6 days, when 0.23 mmol of the products per liter had been produced.

^e NA, not applicable.

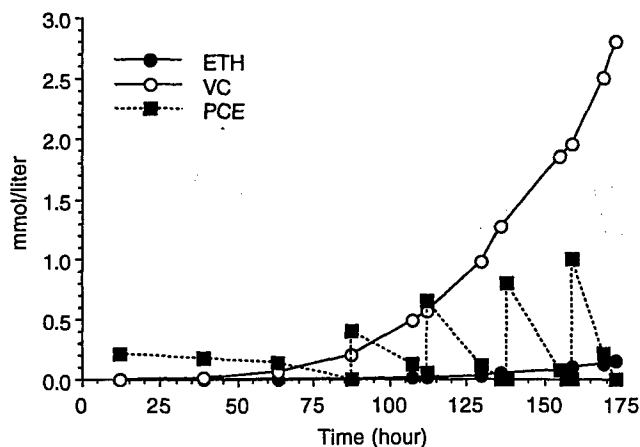


FIG. 2. PCE dechlorination and product formation by a purified H_2 -PCE culture to which consecutively increasing PCE doses were added. The basal medium was amended with 25% (vol/vol) SS, a vitamin solution, and 2 mM Na acetate. The inoculum size was 2% (vol/vol).

The potential requirements for vitamins and SS were also examined for the H_2 -PCE culture. Previous experiments showed that there was significant limitation of dechlorination in the cultures not receiving vitamins compared with the positive control cultures (data not shown). To identify which vitamins were required, an experiment was performed in which 1 of the 10 vitamins present in the standard solution was deleted from each set of duplicate tubes. It was found that cultures with vitamin B_{12} deleted showed dechlorination as poor as that of cultures receiving no vitamins, while deletion of the other vitamins had negligible effects on product formation (data not shown). The requirement for vitamin B_{12} was then examined more closely. Figure 4 shows the effect of adding various amounts of vitamin B_{12} on product formation (mainly VC and ETH) by the culture after 11 days of incubation (no other vitamins were added). Increasing amounts of vitamin B_{12} led to increasing product formation, although the response was not linear. Saturation occurred near 0.05 mg of vitamin B_{12} per liter, at which concentration the cultures performed as well as those receiving all 10 vitamins. In uninoculated cultures, vitamin B_{12} added to concentrations of up to 5 mg/liter did not

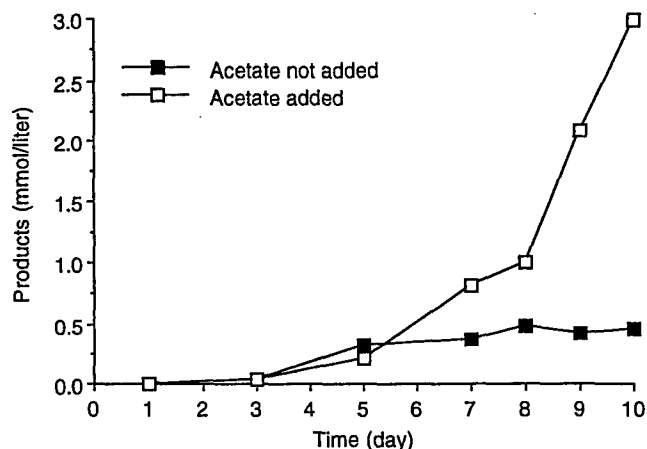


FIG. 3. Product formation (predominantly VC and ETH) from PCE dechlorination by a purified H_2 -PCE culture in the presence or absence of 2 mM Na acetate. The basal medium was amended with 25% (vol/vol) SS and vitamins, and the inoculum was 1% (vol/vol).

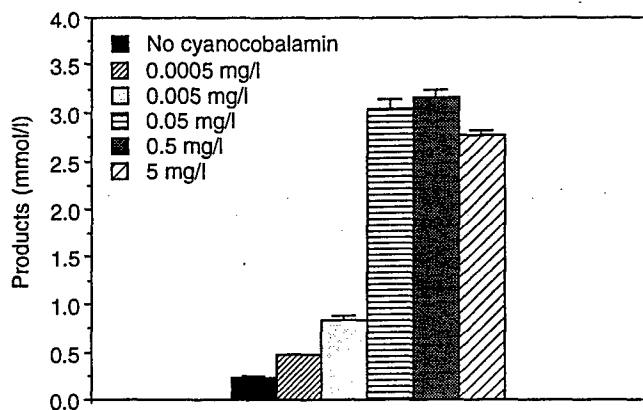


FIG. 4. PCE dechlorination products as measured after 11 days from inoculation (1% [vol/vol]) of the purified H_2 -PCE culture in tubes containing different concentrations of vitamin B_{12} . The basal medium was amended with 25% (vol/vol) SS and 2 mM Na acetate.

catalyze measurable reductive dechlorination of PCE (data not shown).

When SS was not added to the growth medium, the culture did not consume the first dose of PCE within 14 days (Fig. 5), suggesting nutrient limitation. Adding the equivalent of 5% (vol/vol) SS allowed the consumption of four increasing doses of PCE, with little accumulation of ETH and significant accumulation of DCEs and TCE between feedings. Adding the equivalent of 25% (vol/vol) SS, the standard dose used in other experiments, allowed the consumption of multiple and increasing doses of PCE, although there were signs of limitation (residual TCE and PCE) after the last dose. Addition of 50% (vol/vol) SS allowed the best consumption of PCE, with the greatest ETH accumulation, while cultures amended with 100% (vol/vol) SS did not perform as well, showing a decrease in the rate of PCE degradation at the end of the incubation.

Microscopic observations of the methanol-PCE culture and the purified H_2 -PCE culture. In the purified H_2 -PCE culture grown in medium with acetate, SS, and vitamins, the two major morphotypes were irregular cocci and small rods (Fig. 6). Larger rods were also readily observed but were far less nu-

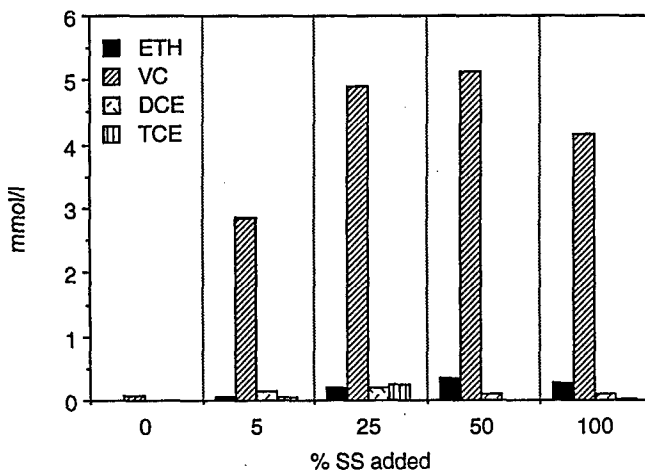


FIG. 5. PCE dechlorination products as measured after 14 days from inoculation (2% [vol/vol]) of the purified H_2 -PCE culture. Tubes were amended with different percentages of lyophilized SS and were given new PCE doses as soon as PCE was consumed. The basal medium was amended with vitamins and 2 mM Na acetate.

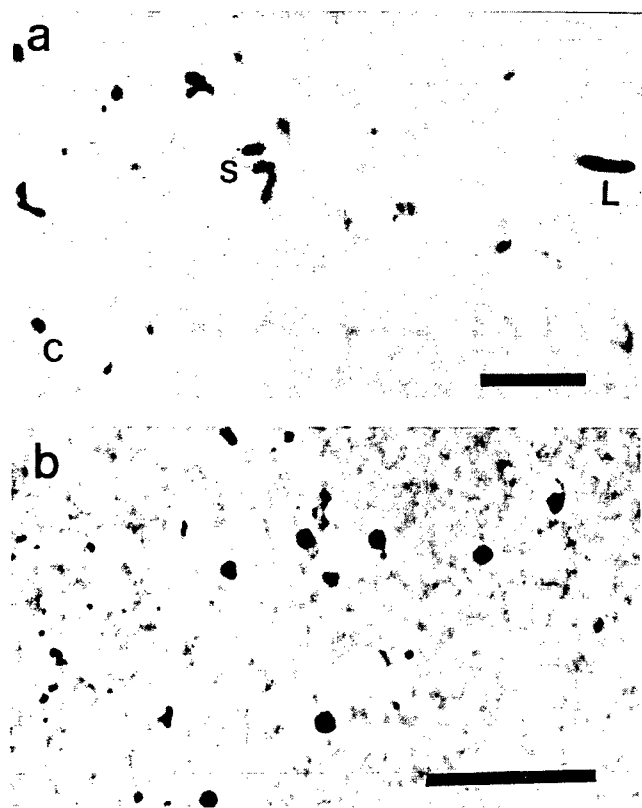


FIG. 6. (a) Photomicrograph of a purified H_2 -PCE culture concentrated 25-fold showing short rods (S), a long rod (L), and irregular cocci (C). (b) Higher-magnification photomicrograph of the concentrated H_2 -PCE culture showing a microscopic field containing many irregular cocci. The microscopy conditions were adjusted to enhance the contrast between the cocci and the background. Bars, 5 μ m.

merous than the previously mentioned morphotypes. The irregular cocci were absent from cultures which did not show PCE dechlorination (data not shown), suggesting their involvement in that process, although more conclusive evidence is needed before the process can be ascribed to any one organism.

DISCUSSION

The results presented here are consistent with the primary prediction of the model of DiStefano et al. (8), in which H_2 (or possibly formate) is the primary electron donor for reductive dechlorination in the methanol-PCE culture (Fig. 1). Specifically, the prediction was borne out that there should be relatively high numbers of methanol-utilizing acetogens and H_2 -utilizing PCE dechlorinators in the culture. Indeed, when tested after several transfers, the H_2 -PCE culture derived from a 10^{-6} dilution of the methanol-PCE culture was no longer capable of using methanol as an electron donor for dechlorination or for acetogenesis (Table 2). Presumably, the methanol-utilizing acetogens were initially present in the 10^{-6} H_2 -PCE dilutions, since they were present in the original methanol-PCE culture at numbers exceeding 10^7 /ml, but were lost after several transfers in medium lacking a substrate for their growth. Acetate, which was present in the medium at 2 mM, also could not serve as an electron donor for dechlorination (Table 2).

The model also predicts low numbers of methanogens in the methanol-PCE culture, and only few H_2 - CO_2 -utilizing meth-

anogens were detected (Table 1). This is in contrast to earlier studies on the methanol-PCE culture, in which the PCE dose was much lower and the culture was actively methanogenic (12) and we found over 10^7 cells of a methanol-utilizing methanogen resembling *Methanosarcina* sp. per ml (30). The lack of evidence of the presence of methanogens and acetogens in the purified H_2 -PCE culture indicates that these organisms were not primarily responsible for the high-rate PCE dechlorination in the original methanol-PCE culture.

H_2 was able to serve indefinitely as the electron donor for reductive dechlorination of PCE to VC and ETH in cultures provided with proper nutritional supplements, in agreement with previous results (8). The degradation of PCE by the H_2 -PCE culture at an increasing rate, as well as our ability to transfer it indefinitely, is indicative of growth of the culture concurrent with the dechlorination process. H_2 and PCE have been shown to serve as an electron donor-acceptor pair for the growth of strain PER-K23 (16) and *D. multivorans* (20) cultures, but in those cases, reductive dechlorination stopped at *cis*-DCE. No evidence of DCE accumulation in this culture was obtained, and neither of the two dominant morphotypes in the purified H_2 -PCE culture, when examined microscopically, resembled strain PER-K23 or *D. multivorans*, so it is likely that a different organism is responsible for PCE dechlorination in this culture.

That a 10^{-6} dilution of the methanol-PCE culture on H_2 -PCE required 35 days (Table 1) to accomplish approximately 20 doublings ($2^{20} \approx 10^6$) suggests a doubling time of less than 2 days for the H_2 -PCE culture, which is consistent with the increasing rate of metabolism in Fig. 2.

The purified H_2 -PCE culture was able to convert PCE to ETH, although the ratio of ETH/VC produced from PCE was much lower than that described for the methanol-PCE culture (7). This is at least partially due to the feeding regimen we used for the H_2 -PCE culture in these experiments, in which we provided a dose of PCE as soon as its depletion was detected. Since PCE can inhibit VC dechlorination (22), the cultures had little opportunity to accumulate ETH.

The purified H_2 -PCE culture could be transferred indefinitely in growth medium supplemented with acetate, SS, and vitamins. The acetate most likely serves as a carbon source for the culture and, as such, supports considerably less growth of potential contaminants than does YE. The optimal amount of SS required was 25 to 50% (vol/vol). It is not clear whether the SS directly provides nutrients required by PCE dechlorinators or whether products of metabolism of SS components by other organisms are required. The SS contributed less than 20 mg of carbon per liter to the culture when added at 25% (vol/vol), and since some portion of it is not readily catabolizable, it should support only a low number of contaminants. The amount of acetate provided by 25% (vol/vol) SS, <0.05 mM, was apparently too low to support the growth of dechlorinators.

The vitamin requirement for PCE dechlorination could be met by adding vitamin B_{12} alone, but since we also needed to add SS in these experiments, it is possible that the SS was providing sufficient quantities of other required vitamins. The vitamin B_{12} requirement is intriguing because it and other corrinoid compounds have been shown to carry out reductive dechlorination of chloro-organics, including chloroethenes, *in vitro* (13, 17, 20). We verified that no reductive dechlorination of PCE occurred in uninoculated medium supplemented with vitamin B_{12} . Still, the requirement for vitamin B_{12} suggests a role in dechlorination in this organism, perhaps as a prosthetic group of a dechlorinating enzyme. The amount required, between 0.005 and 0.05 mg/liter, is considerably greater than the amount (0.001 mg/liter) typically supplied to organisms which

use vitamin B₁₂ for anabolic reactions (3). The vitamin B₁₂ requirement may explain why methanol was the electron donor which led to the best sustained dechlorination in the early studies on this culture (7, 12). Those cultures were amended with YE, which lacks vitamin B₁₂ (3). However, methylotrophic methanogens (5) and acetogens (18) growing on methanol are rich in cobamides, which could be cross-fed to the dechlorinating organisms by lysis and possibly excretion. For example, a *Methanosarcina* culture grown on acetate supported the growth of a vitamin B₁₂-requiring contaminant (25).

These results show that like many other anaerobes, the PCE dechlorinators in this culture are dependent on other organisms for several of their nutritional requirements. Knowledge of some of these requirements has allowed us to simplify the culture medium we use for them, and it is hoped that further unraveling of these requirements will lead to isolation of PCE dechlorinators by providing the nutrients they require to grow axenically without supporting significant growth of contaminants. Knowledge of the nutrition of PCE dechlorinators can also have practical significance. Fennell and Gossett (11) have found in bioreactor studies that the methanol-PCE culture could be switched to butyrate as an electron donor, but only when vitamins, including vitamin B₁₂, were added. Thus, addition of a simple vitamin solution has allowed greater flexibility in electron donor use by a PCE-dechlorinating mixed culture.

ACKNOWLEDGMENTS

This research was supported by the U.S. Air Force's Armstrong Laboratory, Environmental Quality Directorate, Tyndall Air Force Base, Fla., and by the Cornell Center for Advanced Technology (CAT) in Biotechnology, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the National Science Foundation. Xavier Maymó-Gatell was supported by a fellowship from the "La Caixa" Foundation, Catalonia, Spain. Valter Tandoi was supported by NATO senior fellowship 217.24.16.

We thank Tim Anguish for technical assistance; William C. Ghiorse, Lynne Anguish, and Rhea Garen for assistance with photomicroscopy; and Daniel Miller for assistance with organic carbon analysis.

REFERENCES

1. Bagley, D. M., and J. M. Gossett. 1990. Tetrachloroethene transformation to trichloroethene and *cis*-1,2-dichloroethene by sulfate-reducing enrichment cultures. *Appl. Environ. Microbiol.* **56**:2511-2516.
2. Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. *Microbiol. Rev.* **43**: 260-296.
3. Cote, R. J., and R. L. Gherna. 1994. Nutrition and media, p. 155-178. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington, D.C.
4. de Bruin, W. P., M. J. J. Kotterman, M. A. Posthumus, G. Schraa, and A. J. B. Zehnder. 1992. Complete biological reductive transformation of tetrachloroethylene to ethane. *Appl. Environ. Microbiol.* **58**:1996-2000.
5. DiMarco, A. A., T. A. Bobik, and R. S. Wolfe. 1990. Unusual coenzymes of methanogenesis. *Annu. Rev. Biochem.* **59**:355-394.
6. DiStefano, T. D. 1992. Biological dechlorination of tetrachloroethene under anaerobic conditions. Ph.D. thesis. Cornell University, Ithaca, N.Y.
7. DiStefano, T. D., J. M. Gossett, and S. H. Zinder. 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl. Environ. Microbiol.* **57**:2287-2292.
8. DiStefano, T. D., J. M. Gossett, and S. H. Zinder. 1992. Hydrogen as an electron donor for the dechlorination of tetrachloroethene by an anaerobic mixed culture. *Appl. Environ. Microbiol.* **58**:3622-3629.
9. Ensley, B. D. 1991. Biochemical diversity of trichloroethylene metabolism. *Annu. Rev. Microbiol.* **45**:283-299.
10. Fathepure, B. Z., J. P. Nengu, and S. A. Boyd. 1987. Anaerobic bacteria that dechlorinate perchloroethene. *Appl. Environ. Microbiol.* **53**:2671-2674.
11. Fennell, D., and J. M. Gossett. Unpublished results.
12. Freedman, D. L., and J. M. Gossett. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* **55**:2144-2151.
13. Gantzer, C. J., and L. P. Wackett. 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* **25**:715-722.
14. Gibson, S. A., and G. W. Sewell. 1992. Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols. *Appl. Environ. Microbiol.* **58**:1392-1393.
15. Gossett, J. M. 1987. Measurement of Henry's law constants for C₁ and C₂ chlorinated hydrocarbons. *Environ. Sci. Technol.* **21**:202-208.
16. Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder. 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl. Environ. Microbiol.* **59**:2991-2997.
17. Jablonski, P. E., and J. G. Ferry. 1992. Reductive dechlorination of trichloroethylene by CO-reduced CO dehydrogenase enzyme complex from *Methanosarcina thermophila*. *FEMS Microbiol. Lett.* **96**:55-60.
18. Ljungdahl, L. G. 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. *Annu. Rev. Microbiol.* **40**:415-450.
19. Lobo, A. L., and S. H. Zinder. 1988. Diazotrophy and nitrogenase activity in the archaeobacterium *Methanosarcina barkeri* 227. *Appl. Environ. Microbiol.* **54**:1656-1661.
20. Neumann, A., H. Scholz-Muramatsu, and G. Diekert. 1994. Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch. Microbiol.* **162**: 295-301.
21. Sewell, G. W., and S. A. Gibson. 1991. Stimulation of the reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by the addition of toluene. *Environ. Sci. Technol.* **25**:982-984.
22. Tandoi, V., T. D. DiStefano, P. A. Bowser, J. M. Gossett, and S. H. Zinder. 1994. Reductive dehalogenation of chlorinated ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. *Environ. Sci. Technol.* **28**:973-979.
23. Tandoi, V., and S. H. Zinder. Unpublished results.
24. Vogel, T. M., and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Appl. Environ. Microbiol.* **49**:1080-1083.
25. Ward, D. M., R. A. Mah, and I. R. Kaplan. 1978. Methanogenesis from acetate: a nonmethanogenic bacterium from an anaerobic acetate enrichment. *Appl. Environ. Microbiol.* **35**:1185-1192.
26. Widdel, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469-586. In A. J. B. Zehnder (ed.), *Biology of anaerobic microorganisms*. Wiley Interscience, New York.
27. Zeikus, J. G. 1977. The biology of methanogenic bacteria. *Bacteriol. Rev.* **41**:514-541.
28. Zinder, S. H., and T. Anguish. 1992. Carbon monoxide, hydrogen, and formate metabolism during methanogenesis from acetate by thermophilic cultures of *Methanosarcina* and *Methanotherix* strains. *Appl. Environ. Microbiol.* **58**:3323-3329.
29. Zinder, S. H., S. C. Cardwell, T. Anguish, M. Lee, and M. Koch. 1984. Methanogenesis in a thermophilic anaerobic digester: *Methanotherix* sp. as an important acetoclastic methanogen. *Appl. Environ. Microbiol.* **47**:796-807.
30. Zinder, S. H., T. DiStefano, J. Gossett, Y.-L. Juang, R. Vazoller, and T. Anguish. 1991. Preliminary microbiological characterization of an anaerobic enrichment culture which converts tetrachloroethylene to ethylene, abstr. Q-114, p. 295. Abstracts of the 91st General Meeting of the American Society for Microbiology 1991. American Society for Microbiology, Washington, D.C.
31. Zinder, S. H., and M. Koch. 1984. Non-acetoclastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Arch. Microbiol.* **138**:263-272.

Comparative Kinetics of Hydrogen Utilization for Reductive Dechlorination of Tetrachloroethene and Methanogenesis in an Anaerobic Enrichment Culture

CONCORDIA R. SMATLAK AND
JAMES M. GOSSETT*

*School of Civil and Environmental Engineering, Hollister Hall,
Cornell University, Ithaca, New York 14853*

STEPHEN H. ZINDER

*Section of Microbiology, Wing Hall, Cornell University,
Ithaca, New York 14853*

Anaerobic microorganisms that reductively dechlorinate tetrachloroethene (PCE) must often compete with methanogens for H_2 . This study compared the kinetics of H_2 utilization between the two types of organisms at 35 °C under conditions of continuous agitation. Limiting levels of H_2 were administered to 160-mL serum bottles seeded with a PCE/butyric acid enrichment culture; H_2 , methane, and vinyl chloride were tracked over time using headspace samples. Measured half-velocity constants with respect to H_2 — $K_s(H_2)$ values \pm 95% CI—for methanogenesis and dechlorination were 960 ± 180 and 100 ± 50 nM, respectively. Mass-transfer equations were used to calculate aqueous H_2 concentrations at the half-velocity point from headspace measurements. The possible effect on $K_s(H_2)$ values arising from interconversion between H_2 and formate through an active formate/ H_2 lyase system was examined by comparing results from formate-fed and H_2 -fed bottles. Only methanogens in the culture were apparently capable of using formate; hence, the measured methanogenic $K_s(H_2)$ was dependent on which electron donor was administered. The nearly 10-fold difference in $K_s(H_2)$ between methanogens and dechlorinators suggests that the deliberate choice of an electron donor whose fermentation results in a slow, steady, and low-level release of H_2 over time could maximize dechlorination potential while minimizing methanogenic competition for H_2 .

Introduction

Tetrachloroethene (also known as perchloroethylene or PCE) is a commonly used cleaning solvent that has become an all-too-common groundwater pollutant. Recalcitrant under aerobic conditions, PCE has been observed under anaerobic conditions to be reductively dechlorinated to less chlorinated ethenes—trichloroethene (TCE), the dichloroethene (DCE) isomers, and vinyl chloride (VC)—and, in some instances, completely dechlorinated to ethene (ETH) or ethane (1–4). Unfortunately, the degree of dechlorination achieved is inexplicably variable among field sites.

In situ anaerobic biological treatment as well as natural-attenuation strategies have received increased attention due to their potential as cost-effective, permanent solutions for PCE-contaminated subsurface sites. However, success or failure depends on whether the dechlorination is complete or partial. We believe that, among other factors, microbial competition is important in determining the outcome—i.e., whether the organisms responsible for dechlorination are capable of accessing the available pool of necessary nutrients and/or electron donor(s) in the presence of any competitors.

We earlier developed a methanol-fed, anaerobic enrichment culture capable of reductively dechlorinating PCE to VC and ETH. The culture dechlorinated PCE levels of 550 μ M to (mostly) ETH within 2 days, using H_2 formed during the acetogenic metabolism of methanol as the actual, direct electron donor for the sequential process (5, 6). The use of H_2 as a direct donor for dechlorination in this mixed culture was confirmed through later studies with a highly purified culture of the dechlorinating microorganism (7). Though there are likely to be exceptions, it appears that H_2 may generally serve as direct electron donor for dechlorination of PCE, as inferred from soil-microcosm studies (8) and from characterization of the only two isolates thus far reported in the literature—PER-K23 (9, 10) and *Dehalospirillum multivorans* (11).

Our methanol–PCE culture lacked a significant methanogenic presence, due to the inhibitory effect of the administered high level of PCE on methanogens. In later studies in which noninhibitory (ca. 110 μ M) levels of PCE were administered, dechlorination waned as competition for electron donor became severe (12); the methanol-using methanogens and acetogens competed directly for methanol, and the hydrogenotrophic methanogens and H_2 -using dechlorinators competed for what little H_2 was produced indirectly from the acetogenic process or from endogenous fermentation. The ultimate failure of these systems—resulting in increasingly more methanogenesis and little to no dechlorination—emphasized the importance of providing a nonmethanogenic substrate that is fermented directly to H_2 (e.g., ethanol, lactate, or butyrate). In long-term, semicontinuous reactor studies, PCE dechlorination to ETH was sustained by ethanol-fed, lactate-fed, and butyrate-fed systems, although butyrate—whose fermentation to H_2 is thermodynamically limited to relatively low H_2 levels—resulted in the most complete dechlorination (13). In time course experiments with these cultures, the patterns of H_2 formation, PCE depletion, and methanogenesis suggested that dechlorinators could use H_2 at lower levels than could methanogens—as the H_2 pool was depleted,

* Corresponding author telephone: 607-255-4170; fax: 607-255-9004; e-mail address: jmg18@cornell.edu.

dechlorination of PCE continued while methanogenesis ceased. These results suggested that there was an inherent difference in affinity for H_2 between the dechlorinators and methanogens of this culture—a difference that might be exploited in bioremediation strategies attempting to optimize the delivery of electron donor to the dechlorination process.

In this paper, we compare the kinetics of H_2 use by methanogens and dechlorinators in a mixed-culture system, by direct measurement of their respective half-velocity constants with respect to H_2 . Aqueous H_2 levels in the bulk solution were estimated by accounting for mass transfer across the gas/liquid interface and employing simple mass-transfer theory. Complications associated with formate/ H_2 lyase activity and its influence on the $K_s(H_2)$ for both methanogenesis and dechlorination were evaluated by comparing the results of both H_2 -fed and formate-fed systems. Results suggest that the deliberate choice of an electron donor with slow, steady release of H_2 over time could minimize methanogenic competition in this mixed culture. These results have implications for the development of *in situ* bioremediation strategies.

Materials and Methods

Chemicals and Stock Solutions. PCE (99.9%; Eastman Kodak Co.), formate (92%; Fisher Scientific Co.), and H_2 (99.95% minimum purity from Linde Specialty Gases and Equipment) were used as culture substrates and analytical standards. TCE (99%; Fisher Scientific Co.), H_2 (1% in N_2 from Airco, Inc.), VC (99%; Matheson Gas Products), CH_4 (Scientific Gas Products), and ETH (Matheson Gas Products) were obtained for the development of analytical standards. HPLC-grade H_2O (Fisher Scientific Co.) and concentrated, analytical-grade H_2SO_4 (Mallinckrodt, Inc.) were used to develop a 0.013 N solution for the mobile phase of the HPLC.

Yeast extract (Difco Laboratories) was used to develop an anoxic, 50 g/L stock solution that served as a nutrient source for the culture. Pre-fermented yeast extract (FYE) stock was prepared by inoculating the solution with the source culture and waiting 10 days before use. The FYE was presumed to be a nutrient source with less significant electron-donor content than unfermented yeast extract.

PCE-laden basal medium (PCE-LBM) was prepared by setting up a saturated anaerobic solution of approximately 100 μ L of PCE in 150 mL of basal medium (6). PCE saturation occurs at about 200 mg/L (≈ 1.21 mM). A total of 10 mL of PCE-LBM therefore corresponds to about 12 μ mol of PCE, or 96 μ equiv, because the complete reductive dechlorination of PCE to ETH requires 8 equiv/mol. The PCE-LBM bottles were placed on an orbital shaker at 35 °C for 1 day, followed by at least 2 days of quiescence before using.

Cultures and Enrichment Procedures. A low-PCE/butyrate enrichment culture was developed by Fennell (13) using seed from the DiStefano et al. (5) methanol-fed culture. The fermentation of butyrate—a nonmethanogenic substrate—to acetate and H_2 provided the necessary electron donor for sustained dechlorination at lower PCE levels (~ 110 μ M). With routine feeding of butyrate and PCE every 2 days at a ratio of 2:1 (in terms of electron equivalents, with butyrate defined as having 4 equiv/mol, based on its fermentation to acetate and H_2 rather than its oxidation to CO_2), PCE was dechlorinated to VC and mostly ETH.

The source low-PCE/butyrate culture was grown in a 9.1-L stirred reactor (5.7-L liquid volume) at 35 °C and operated with a nominal hydraulic retention time of 40 days. The basal medium (6) and reactor (14) have been described previously. Every 2 days, the culture was fed 110 μ mol of PCE and 440 μ mol of butyrate/L of culture; it was also administered 2.3 mL of a 50 g/L anoxic FYE stock (resulting in a 20 mg/L incremental reactor concentration). Every fourth day, the reactor was purged of volatile compounds with a 70% N_2 /30% CO_2 gaseous mixture [scrubbed of trace levels of oxygen by first passing through a titanous chloride/sodium bicarbonate/sodium citrate solution (15)], 10% of the culture was wasted and replaced with fresh basal medium, and 2.9 mL of a vitamin solution was added, in addition to the normal 2-day feeding regimen. The vitamin solution was that described by Balch et al. (16), except that it was concentrated 10-fold with respect to all components in all experiments, excluding vitamin B_{12} , which was concentrated 100-fold to 0.01 g/L in experiments III–VI since studies of the purified dechlorinating culture have indicated a high vitamin B_{12} requirement (7).

Preparation for Bottle Experiments. Each bottle was prepared 4 days prior to its respective kinetic experiment, as follows: 100 mL of inoculum from the low PCE/butyrate source culture was anaerobically delivered to 160-mL serum bottles by a Unispense-II pump (Wheaton Industries). A Teflon-coated butyl rubber septum (Wheaton Industries) sealed in place with an aluminum crimp cap was used to maintain anaerobic conditions. All septa were autoclaved before using so as to thermally strip volatile organics which would possibly interfere with analyses.

The feeding protocol on day –4 was similar to that of the source culture so as to maximize the probability of the success of transfer—12 μ mol of neat PCE, 48 μ mol of butyric acid, and the corresponding FYE and vitamin doses were delivered. On day –2, PCE and vitamin solution were added as usual, but H_2 (96 μ mol) was the administered electron donor, and FYE was withheld from all bottles. Although FYE apparently contains important nutritional factors for the long-term sustenance of PCE dechlorination in this culture, in short-term studies such as these, the FYE was withheld to avoid the possibility of providing acetate that would support undesired activity of acetotrophic methanogens, known to be a significant presence in the source culture. In experiment I, the cultures were in fact examined on day –1 for the presence of acetate, which was found to be below the detection limit of 20 μ M. In experiments II–VI, “regular” or unfermented yeast extract was provided on day –4 with the acetate level on day –1 again measured to be negligible. A control experiment described below, confirmed that acetotrophic methanogenesis was insignificant in these experiments.

After the initial preparation was complete, all bottles were maintained in an inverted position (with the liquid in contact with the septum to minimize losses) on an orbital shaker at 165 rpm, 35 °C, as would be the case for the entirety of each time course experiment.

Experimental Procedure. On both day –2 and day 0, the headspaces of all bottles were analyzed to ascertain health, as evidenced by PCE dechlorination. After analysis on day 0, all bottles were purged; during this purge, 10 mL of culture was wasted via syringe. The bottles were then recapped, and 10 mL of either PCE-LBM (for those bottles to be evaluated for dechlorination and methanogenesis) or

TABLE 1
Feeding Regimens for Respective Bottle Studies

bottle set	electron donor*	PCE added (μmol)	ED/PCE (equiv/equiv)	μequiv of ED	μmol of ED
Experiment I: Dechlorination and Methanogenesis, H₂-Fed					
1	H ₂	12	2:1	192	96
2	H ₂	12	3:1	288	144
3	H ₂	0	2:0	192	96
Experiment II: Dechlorination and Methanogenesis, H₂-Fed					
1	H ₂	12	2:1	192	96
2	H ₂	0	2:0	192	96
Experiments III and V: Methanogenesis Only, H₂-Fed vs Formate-Fed					
1	H ₂	0	2:0	192	96
2	formate	0	2:0	192	96
Experiments IV and VI: Dechlorination and Methanogenesis, H₂-Fed vs Formate-Fed					
1	H ₂	12	2:1	192	96
2	formate	12	2:1	192	96

* Abbreviated ED thruout table.

plain basal medium (for those bottles intended for study of methanogenesis only) was added. The usual dose of vitamin solution (50 μL) was also added to each experimental bottle along with the appropriate level of electron donor.

Table 1 shows the required additions of PCE and electron donor (ED) corresponding to the appropriate experiment (the Roman numeral corresponds to the chronological order in which the experiments were performed). Both H₂ and formate provide 2 equiv/mol. The entire series of experiments took place over a 6-month period. All "bottle sets" consisted of at least duplicate bottles.

One potential complication and ramification of H₂ being a gaseous substrate was excessive overpressure in the bottles. To prevent the potential for increased leakage under such circumstances, any overpressure more than 1.0 mL in the bottles prior to the addition of H₂ was removed.

A seventh experiment was performed in which methane production and dechlorination were monitored from unfed controls (neither H₂ nor formate was delivered to the bottles) after a standard 4-day setup regimen. On the fourth day, PCE-LBM (or plain basal medium, where appropriate) and vitamin solution were added, and the yeast extract was withheld. This was done to evaluate the possible acetotrophic methanogenic contribution to the overall methane formed, which, if significant, would render the kinetic constants derived from the headspace methane measurements meaningless, insofar as it was not possible to distinguish the methane formed by hydrogenotrophic methanogens from that of acetotrophic methanogens.

For each kinetic experiment, "time zero" was defined when the appropriate amount of H₂ or formate was added to each bottle.

Analytical Methods. The total masses of PCE, TCE, DCEs, VC, CH₄, ETH, and H₂ per bottle were measured using a gas chromatographic (GC) technique developed by Freedman (2) and DiStefano (5), involving two columns, two switching valves, two flame ionization detectors (FIDs), and a reduction gas detector (RGD) for low levels of H₂ (Trace Analytical, Inc.). Single 0.1-mL headspace injections were made to the GC network via a gas-tight locking syringe. The carrier gas was N₂ (prepurified, Linde Specialty Gases and Equipment) that had been further purified of H₂

contamination by pretreatment through a catalytic combustion filter (Trace Analytical, Inc.) followed by a molecular sieve (Supelco, Inc.).

For calibration of lower H₂ levels on the RGD, the standard bottles (160-mL serum bottles containing 100 mL of distilled, deionized water) were first purged with the pre-purified N₂ carrier gas stream. H₂ amounts ranging from 0 to 4 μmol were added; particularly low levels of H₂ were delivered from a 1% in N₂ mixture. The bottles were placed in the 35 °C orbital shaker/water bath and allowed to equilibrate for 1 h; 0.1-mL headspace samples were then assayed.

During the first two hours of an experiment—when H₂ levels were above the range of the RGD (i.e., >4 μmol /bottle)—a separate 0.5-mL injection was made to assay H₂ using a thermal conductivity detector (TCD). The column was 3.2 mm by 3.05 m stainless steel packed with 100/120 Carbosieve S-II (Supelco, Inc.) and was isothermally housed at 150 °C.

Over the first two hours of an experiment, liquid samples were also removed to measure formate levels while they were above the detection limit (1 mg/L) of the diode-array detector of the HPLC (Hewlett Packard 1090). Formate was analyzed using 100- μL injections to a 300 mm \times 7.8 mm HPX-87H ion-exclusion column (BioRad) and a 0.013 N H₂SO₄ mobile phase at 0.7 mL/min, ambient temperature. Sample preparation included filtration of 0.5-mL culture through a 0.45- μm filter disc (Gelman Sciences) into a 2-mL autosampler vial (Supelco, Inc.), which contained 10 μL of 6 N H₂SO₄, followed by refrigeration. Standard curves employing peak area responses were developed.

A volatile suspended solids (VSS) analysis was performed after each experiment was completed to provide a surrogate measure of biomass. Culture contents were filtered through previously washed, ignited, and tared GF/F glass microfiber filters (Whatman International Ltd.); drying temperature was 105 °C (1 h); ignition was performed at 550 °C (15 min).

Data Analysis and Interpretation. Determining the affinity of an organism for H₂ is analogous to examining its half-velocity constant with respect to H₂— $K_s(\text{H}_2)$ —essentially the reciprocal of the affinity for H₂. As normally defined and used, $K_s(\text{H}_2)$ corresponds to the bulk solution concentration of H₂ that supports half-maximum uptake rates. Most commonly, $K_s(\text{H}_2)$ values are obtained by measuring the H₂ depletion over time in batch-fed systems. However, several complications arise in the measurement and interpretation of $K_s(\text{H}_2)$ values.

One complication is derived from using headspace measurements (instead of aqueous measurements) of H₂ at the point of half-maximum velocity in batch-fed systems. This is due to interphase mass-transfer limitations between the headspace and the liquid. Any rate of change in the headspace concentration necessarily means disequilibrium between the phases (17). Gas-phase concentrations will always lag behind aqueous-phase concentrations because of gas-to-liquid mass-transfer limitations. Thus, dissolved H₂ concentrations will be overestimated if calculated from measured gaseous concentrations using an equilibrium assumption (18).

A second, fundamental difficulty with interpreting $K_s(\text{H}_2)$ values stems from the practical definition of $K_s(\text{H}_2)$ as being the *bulk solution*—and not the *intracellular*—concentration of H₂ supporting half-maximum utilization rate. A gradient will necessarily exist between these two concentrations. When H₂-fed, microorganisms will nor-

mally experience higher bulk solution than intracellular H_2 concentrations, supporting the necessary mass flux of H_2 to the cell, equivalent to H_2 uptake rate (19). But the relationship between bulk solution and intracellular concentrations will necessarily vary with uptake rate; a larger gradient will develop at higher uptake rates. This ordinarily presents little concern, since the gradient at the half-velocity point would be expected to be relatively constant, allowing at least some reasonably reproducible value of practical $K_s(H_2)$ to be estimated.

A further complication arises in the determination of the $K_s(H_2)$ values where the possibility exists that a formate/ H_2 lyase system may be active. Common in anaerobes, the formate/ H_2 lyase system allows for the interconversion of formate and H_2 and is dependent only on the bicarbonate concentration; it can be found in about half of the known species of methanogens (20). Formate thus serves as a H_2 reservoir and can complicate the determination of $K_s(H_2)$ values in those microorganisms possessing the system through effects on the gradient between bulk solution and intracellular H_2 levels. To illustrate with an extreme example: if the lyase equilibrium is rapid enough—i.e., if it is functioning at a rate faster than H_2 utilization by the cell—and the system is formate-fed, microbes containing the lyase system could form H_2 fast enough to cause H_2 transport from the cell, reversing the sign of the normal H_2 gradient. This, in turn, would cause underestimation of the apparent $K_s(H_2)$ for formate-utilizing anaerobes, because the bulk concentration would be lower (rather than higher) than the actual, intracellular H_2 concentration of the microbe. (It should be emphasized, however, that the presence of the formate/ H_2 lyase system in the consortium should have no such effect upon those members that do not possess it.)

Dechlorination of PCE can be observed by inspection of either PCE depletion or dechlorination product formation. Under the conditions employed in these experiments (i.e., relatively short-term studies, with high initial H_2 levels and significant methanogenic competition), the only significant dechlorination product detected was VC. Since it is generally more precise to monitor the formation of small quantities of product than to monitor small depletions of a relatively large added quantity of PCE, dechlorination was assayed exclusively by the observation of VC production.

To find the half-velocity constant of H_2 with respect to dechlorination or methanogenesis, the following assumptions and procedures were invoked: (1) Systems were kinetically "swamped" with PCE, such that H_2 would deplete first. (2) It was assumed that there was no mass-transfer limitation on VC data or methane data (that is, that the alleged total mass in a bottle—estimated by assuming equilibrium partitioning of the measured headspace concentration—was equal to the actual total mass in the bottle). This was demonstrated through the use of a dynamic computer model that incorporated the mass-transfer coefficients of these compounds into an overall model for the degradation of PCE and the production of methane [described elsewhere (21)]. (3) Times at which the velocity was half-maximum for VC and methane productions were noted. (4) Since dynamic modeling demonstrated that, under most conditions, H_2 headspace data were mass-transfer limited (21), the concentrations of H_2 in the liquid phase at half-velocity points were estimated using the following equation:

$$V_g \frac{dC_g}{dt} = K_1 a (C_w - C_w^*) V_w \quad (1)$$

where V_g is the volume of the headspace, 0.060 L; C_g is the measured concentration of H_2 in gas phase at time t ; dC_g/dt is the observed rate of decrease in C_g at time t ; $K_1 a$ is the overall mass-transfer coefficient for H_2 (measured as described below), 69.29/h; C_w is the actual concentration of H_2 in liquid at time t ; C_w^* is the hypothetical concentration of H_2 in liquid if the liquid were at equilibrium with the measured headspace concentration; and V_w is the volume of liquid phase in bottle, 0.100 L. The difference $(C_w - C_w^*)$ represents the instantaneous driving force for mass transfer at any time t , where

$$C_w^* = \frac{C_g}{H_c} \quad (2)$$

and H_c is the pseudo-dimensionless Henry's constant [52.7 at 35 °C (22)]. Therefore, if dC_g/dt and C_g at time t are known, C_w^* can be easily calculated, and the true aqueous concentration (C_w) can be estimated (since all other variables are known or can be measured).

Thus, for each bottle, the VC and methane data vs time were plotted (exemplified by Figure 1). From such plots, the following were estimated: the maximum velocity and the time at which the slope was closest to half-maximum velocity ($t_{1/2 \text{ max}}$). The corresponding plot of headspace H_2 (i.e., C_g) vs time was empirically fit by a polynomial equation (exemplified by Figure 2). The C_g value corresponding to the earlier determined $t_{1/2 \text{ max}}$, along with the slope (dC_g/dt) of the tangent to the H_2 curve at that time (as expressed by the first derivative of the polynomial equation) were noted. These data enabled the calculation of the half-velocity constants—i.e., the actual concentrations of H_2 in the liquid phase at those points (C_w), according to the method described above. The hypothetical values of H_2 in the liquid phase based on headspace measurements were up to 12 times the actual aqueous concentrations, emphasizing the necessity of this mass-transfer-based determination.

The half-velocity constant is conventionally identified as K_s with respect to H_2 , for dechlorination or methanogenesis as appropriate, where the full model for PCE dechlorination in terms of VC production is expressed as follows:

$$\frac{dVC}{dt} = \frac{kXPCE}{K_{s(PCE)} + PCE} \left(\frac{C_{w(H_2)}}{K_{s(H_2)} + C_{w(H_2)}} \right) \quad (3)$$

where k is the maximum specific substrate utilization rate; X is the biomass; and $K_{s(PCE)}$ is the half-velocity constant of dechlorination with respect to PCE.

Equation 3 makes clear the importance of nonlimiting PCE throughout the experimental run; with such levels of PCE, the first portion of the above equation [$PCE/(K_{s(PCE)} + PCE)$] is essentially unity, since the half-velocity constant of dechlorination with respect to PCE is very small (on the order of 0.5 μM) (21, 23).

Therefore, the three ratios of electron donor:PCE (on an electron equivalent basis)—2:1, 3:1, and 2:0—investigated in these experiments were chosen to provide sufficient data for the determination of the maximum rate and time of half-velocity with respect to H_2 —the most crucial step in

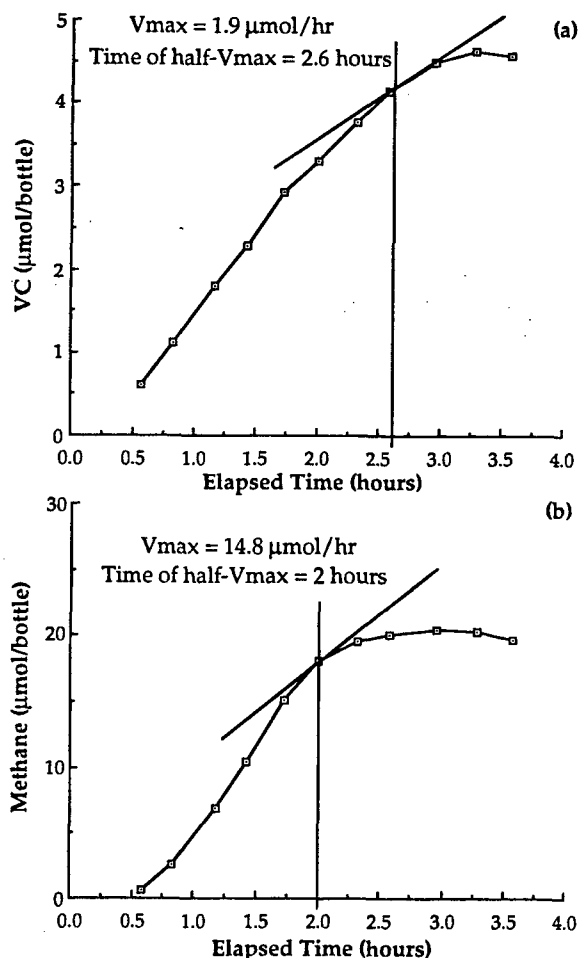


FIGURE 1. Sample plots illustrating the determination of maximum velocity and time of half-maximum velocity of (a) VC production and (b) methanogenesis, bottle VI-2B.

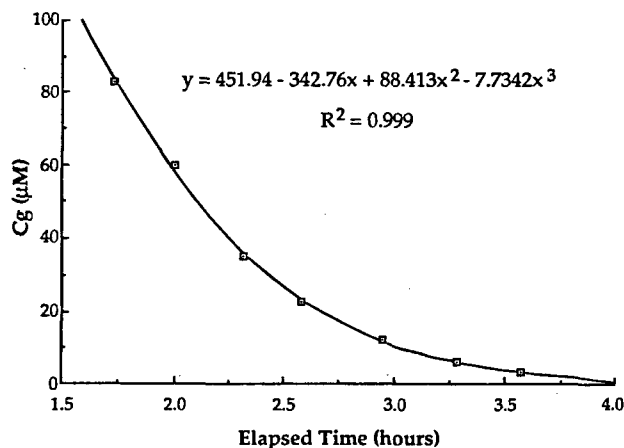


FIGURE 2. Sample plot illustrating a polynomial fit to H_2 data in bottle VI-2B in the time region of half-maximum velocity of dechlorination.

the derivation of each $K_s(H_2)$ —with PCE remaining even after the electron donor was spent.

K_a Determination. Mass-transfer coefficients for VC, PCE, and H_2 were experimentally measured in sterile, 160-mL serum bottles containing 100 mL of anaerobic basal medium at 35 °C. While details are provided elsewhere (21), the method involved the initial addition of aqueous stocks of VC, PCE, or H_2 to the bottle's aqueous phase, with subsequent monitoring of the rate of accumulation in the gaseous phase while the bottles were agitated in an orbital

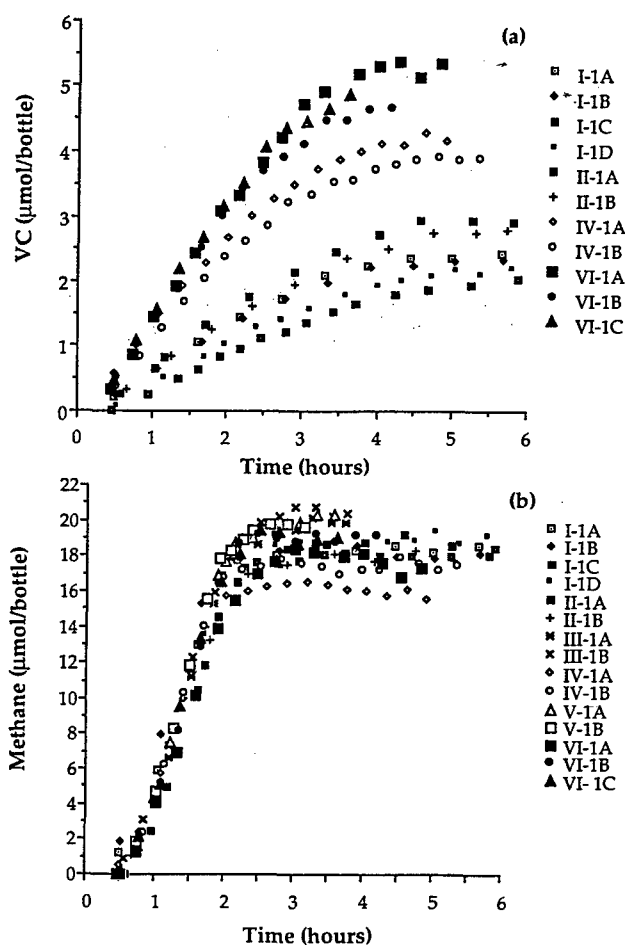


FIGURE 3. (a) Vinyl chloride and (b) methane vs time for all H_2 -fed bottles performed over a 6-month period.

shaker at 165 rpm—the same experimental conditions as in the biokinetic experiments. The K_a values (per hour) were found as follows: VC, 42.8; PCE, 34.2; H_2 , 69.3. Using these values, a general relationship between K_a and the liquid diffusivity (D_l) of each compound was developed and was found to correspond closely to $K_a \propto (D_l)^{1/2}$, the dependence predicted by several gas-transfer models for agitated systems (24). This correlation allowed estimation of the K_a for methane—50 per hour—from a liquid diffusivity of $3.35 \times 10^{-5} \text{ cm}^2/\text{s}$ (25).

Results

Culture Stability. VC and methane time course data from all H_2 -fed experiments conducted over the 6-month analysis period were grouped together and are depicted in Figure 3. From these plots, it is obvious that—while the maximum rate of methanogenesis did not vary systematically with time (ranging from approximately 12 to 16 μmol/h through all experiments)—the maximum rate of dechlorination nearly tripled over the course of these studies (increasing from 0.66 μmol/h in experiment I to 1.9 μmol/h in experiment VI), and the percentage of reducing equivalents routed to dechlorination doubled (from 7.7 to 15.4%). The corresponding decrease expected in methanogenic activity was not apparent, probably because the proportion of methanogenic electron donor use was so high initially (92%); the doubling of reducing equivalents routed to dechlorination would have resulted in a mere 8% drop in the reducing equivalents routed to methanogenesis. Since the total biomass concentration was essentially constant for

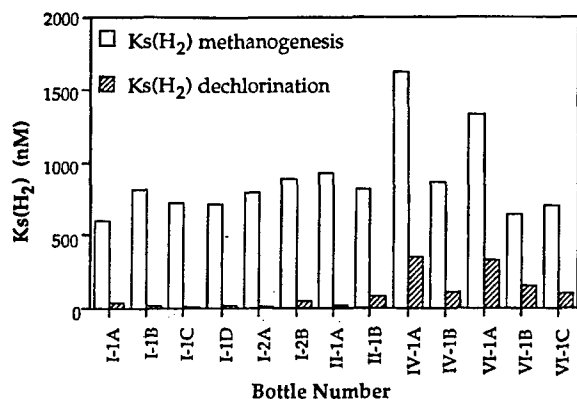


FIGURE 4. Bottle-by-bottle comparison of $K_s(H_2)$ for methanogenesis versus $K_s(H_2)$ for dechlorination.

each study (ca. 80 mg of VSS/L), the increased dechlorination activity is likely the result of an increased dechlorinating fraction of total biomass. This population was perhaps bolstered by the higher dose of vitamin B₁₂ administered to the culture in experiments III–VI.

Because of the change in dechlorination activity over the period of experimentation, it was important to group the data properly when drawing conclusions from the derived $K_s(H_2)$ values for dechlorination. However, because the change in methanogenesis activity over time was not deemed significant, time was not regarded as an important factor when considering the methanogenic $K_s(H_2)$ values.

Bottle Studies. Before $K_s(H_2)$ values were evaluated, it was necessary to determine the possible acetotrophic contribution to methanogenesis. A control experiment was run in which two sets of bottle pairs were subjected to the standard 4-day setup regime and then administered no electron donor (but fed the normal vitamin solution dose)—one set with PCE and the other without it. In this experiment, slow PCE dechlorination was observed from endogenous sources of H₂, but methane levels never rose above the threshold of the FID—estimated to be ca. 0.1 μ mol of methane/bottle (data not shown). These results are consistent with the assumption that there was no significant acetotrophic methanogenic interference in experiments I–VI, and thus the methane data upon which the $K_s(H_2)$ values for methanogenesis were based can be assumed to have been exclusively hydrogenotrophic.

$K_s(H_2)$ values for dechlorination and methanogenesis were compared by examining the results of only those bottles that had both processes occurring simultaneously—i.e., only those bottles that were administered both H₂ and PCE. Although additional data was available from other bottles— $K_s(H_2)$ values from bottles exhibiting methanogenesis only, for example—this limited comparison removed some of the experimental variability among bottles in terms of minor differences in pH, biomass, or other environmental factors. Also, this comparison eliminated time as a variable factor since each bottle contributed a half-velocity constant for both dechlorination and methanogenesis. Figure 4 shows the difference for each bottle between the two types of $K_s(H_2)$. The average ($\pm 95\%$ confidence interval) was 100 ± 60 nM for dechlorination and 880 ± 160 nM for methanogenesis. A two-tailed *t*-test demonstrated that the more than 8-fold difference between the $K_s(H_2)$ for dechlorination and that for methanogenesis was significant at the 95% confidence level. The time course data of VC production and methanogenesis are shown for

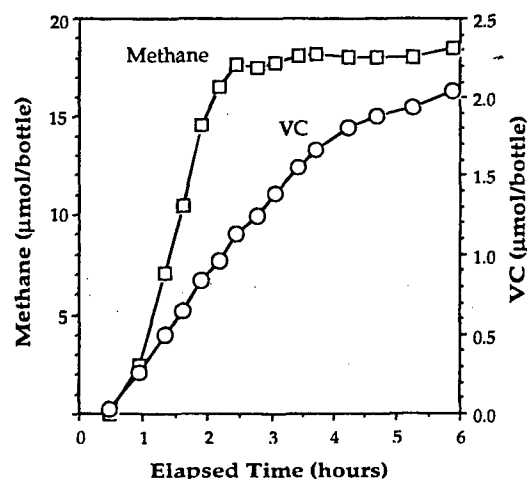


FIGURE 5. Typical H₂-fed and PCE-fed bottle time course of VC and methane productions, low-PCE/butyrate culture.

a typical H₂-fed bottle in Figure 5. Note that in this bottle (as in every bottle exhibiting both dechlorination and methanogenesis), dechlorination continued after methanogenesis had ceased, the expected result of this difference in H₂ affinity between the two types of microorganisms.

The $K_s(H_2)$ values for methanogenesis in the presence and absence of PCE were compared to determine if the administered PCE level of 110 μ M adversely affected the methanogenic kinetics. It was intended that this comparison be accomplished by performing a one-sided *t*-test (appropriate because the hypothesis to be tested was that PCE might increase $K_s(H_2)$ for methanogenesis.) Figure 6 displays the data from both groups in both H₂- and formate-fed systems. In H₂-fed systems, the mean ($\pm 95\%$ CI) $K_s(H_2)$ value for methanogenesis in the presence of PCE was 890 ± 190 nM; in the absence of PCE, it was 1120 ± 420 nM. The corresponding values in formate-fed systems were 520 ± 80 and 620 ± 160 nM, respectively. Since the mean $K_s(H_2)$ values for methanogenesis in the presence of PCE were actually lower than those values in the absence of PCE, no one-sided *t*-test was required to reject the hypothesis that PCE adversely affected methanogenesis. (In fact, a two-tailed *t*-test failed to detect significant difference at the 95% level.) This result allowed the pooling of the methanogenic half-velocity constants (with and without PCE) when the best estimate was later calculated.

The effect of formate on methanogenic kinetics was determined by comparing the mean $K_s(H_2)$ for methanogenesis in H₂-fed systems to that in formate-fed systems. Since it was determined that the presence of PCE did not significantly affect the methanogenic half-velocity constant, systems with and without PCE were grouped in this comparison. The results are displayed in Figure 7; the mean ($\pm 95\%$ CI) $K_s(H_2)$ for methanogenesis in H₂-fed systems was 1250 ± 300 nM, and in formate-fed systems, it was 570 ± 90 nM. A two-tailed *t*-test on the means of both systems concluded with 95% confidence that the half-velocity constants were different.

Figure 8 shows a plot of the formate/H₂ ratios versus time for H₂- and formate-fed bottles over the period when formate measurements were measurable and least subject to imprecision (i.e., at levels higher than 5 mg/L). The disequilibrium between aqueous H₂ and formate (at least in bulk solution) over time was quite apparent. Although this period does not include the methanogenic half-velocity point, it does not appear that the ratios were converging

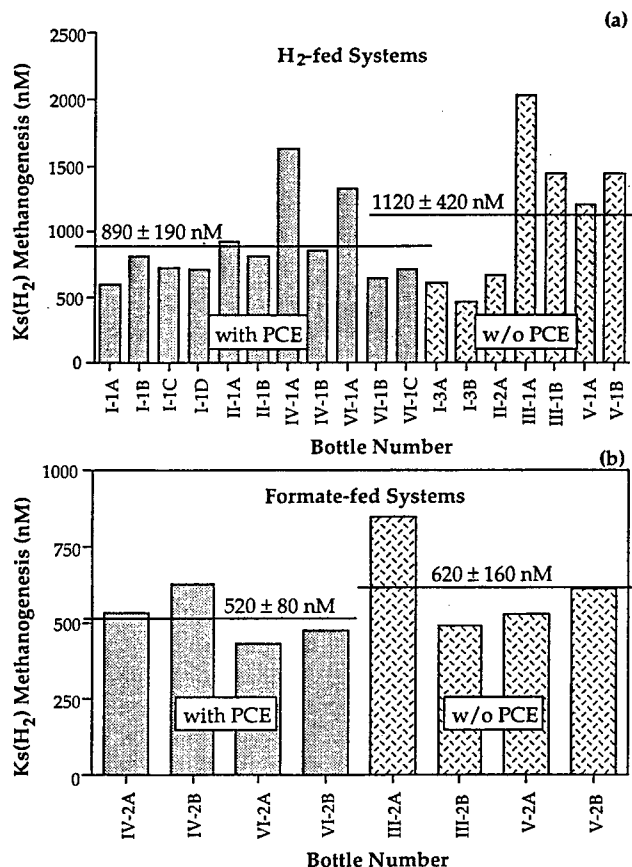


FIGURE 6. Comparison of the $K_s(H_2)$ values for methanogenesis in (a) H_2 -fed and (b) formate-fed systems in the presence and absence of the administered level of PCE ($110 \mu M$). The solid lines mark the averages for each category (\pm values are 95% confidence intervals).

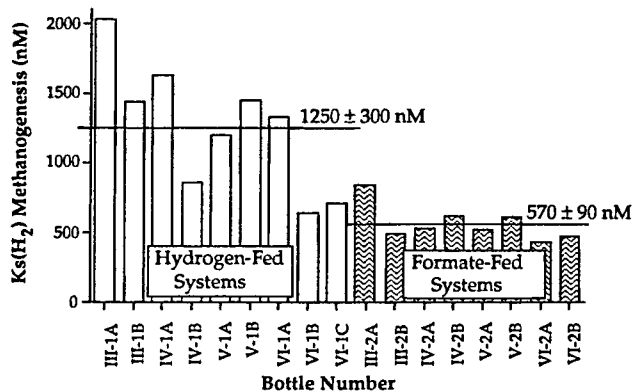


FIGURE 7. Comparison of the $K_s(H_2)$ values for methanogenesis in H_2 -fed versus formate-fed systems. The solid lines mark the averages for each system (\pm values are 95% confidence intervals).

quickly enough that they would have reached a common value by the time the half-velocity point was reached (ca. 2 h). It seems reasonable therefore to attribute the difference in the methanogenic half-velocity constants between H_2 -fed and formate-fed systems or even variability among bottles in the same group (particularly H_2 -fed, with a coefficient of variation among bottles of 36%) to differences in formate/ H_2 ratio. Furthermore, the significant difference in apparent $K_s(H_2)$ between H_2 - and formate-fed bottles suggests that the methanogens in our culture were able to use either substrate to reduce CO_2 to methane.

To determine the effect of formate on the kinetics of PCE dechlorination, the dechlorination half-velocity constants of H_2 -fed and formate-fed systems were compared;

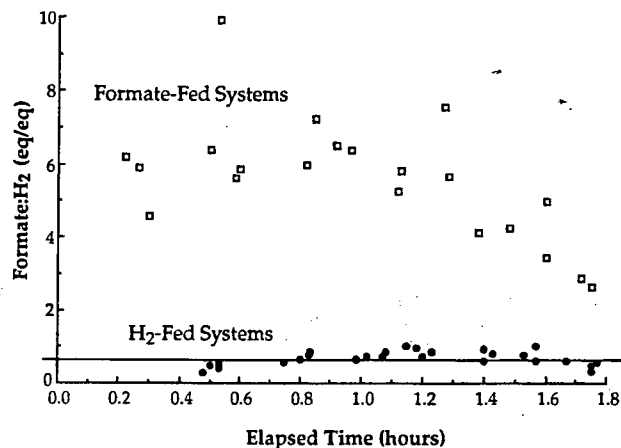


FIGURE 8. Formate/ H_2 ratios (aqueous-phase) versus time for both H_2 -fed and formate-fed systems.

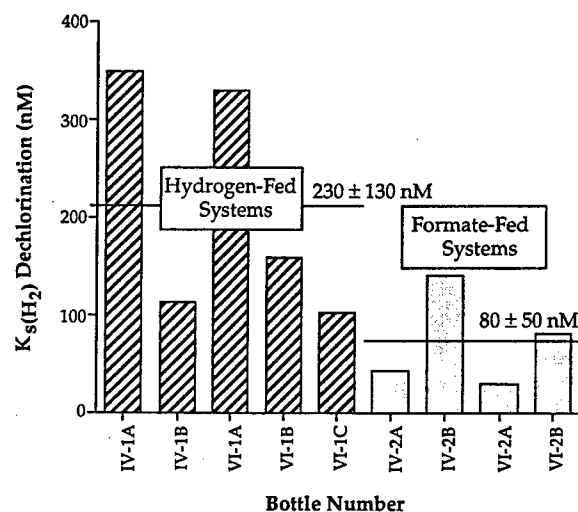


FIGURE 9. Comparison of $K_s(H_2)$ values for dechlorination in H_2 -fed versus formate-fed systems. The solid lines mark the averages for each system (\pm values are 95% confidence intervals).

changes over time within the culture were eliminated as a factor by observing bottles of both types in each experiment. The mean $K_s(H_2)$ for dechlorination in H_2 -fed systems was 230 ± 130 nM, and in formate-fed systems, it was 80 ± 50 nM; these results are represented graphically in Figure 9. Although the mean $K_s(H_2)$ values for dechlorination appeared to depend upon whether H_2 or formate was fed, a two-tailed t -test failed to support a conclusion that the difference was statistically significant at the 95% level.

This result (weakly) corroborates what has been observed more directly by us in unpublished studies—that formate cannot be directly utilized to dechlorinate PCE by the organisms in our culture. Highly purified cultures derived from the same source as these experiments [i.e., the high-PCE/methanol enrichment of DiStefano et al. (5)] when fed formate and ampicillin—which killed contaminating organisms—could not dechlorinate PCE (26). If the dechlorinator could use formate or H_2 , the resulting apparent $K_s(H_2)$ values would be expected to differ between H_2 - and formate-fed systems, dependent on the formate/ H_2 ratio at the half-velocity point, as found in the methanogenic case.

Because the methanogenic half-velocity constants for H_2 - and formate-fed systems were significantly different, only the H_2 -fed system data were pooled to estimate the apparent $K_s(H_2)$ value for methanogenesis. Thus, from the

20 bottles fed H_2 (whether or not they were also administered PCE), the mean apparent $K_s(H_2)$ value for methanogenesis (and 95% confidence interval) was estimated at 960 ± 180 nM. Because there was no difference between the H_2 - and formate-fed systems with regard to dechlorination $K_s(H_2)$ values, it was possible to pool all 17 of the $K_s(H_2)$ values for dechlorination found in this series of experiments to yield an overall average of 100 ± 50 nM.

Discussion

The $K_s(H_2)$ for dechlorination in the butyric acid enrichment culture (approximately 100 nM) was much less than the $K_s(H_2)$ for methanogenesis (approximately 1000 nM). The apparent intracellular interconversion of H_2 and formate by the methanogen in this culture complicates the interpretation of $K_s(H_2)$ values for methanogenesis. However, the overall result of such a high relative $K_s(H_2)$ for the methanogen in this culture provides an indication that dechlorination could be enhanced by management of H_2 delivery—that is, through the deliberate choice of an electron donor whose fermentation results in a slow, steady, and low-level release of H_2 over time. These results suggest that such an electron donor could maximize dechlorination potential while minimizing the methanogenic competition for H_2 . These results, in fact, serve to partially explain the success of butyric acid as an electron donor in mixed-culture dechlorination (13). The slow release of H_2 that it provides helps to minimize the competition for H_2 by methanogens. Further research comparing the degradation kinetics and H_2 formation from other substrates might reveal an even better, more efficient H_2 -delivery strategy for the benefit of PCE dechlorination.

These experimental results demonstrate the importance of invoking mass-transfer theory to estimate the aqueous levels of H_2 at the half-velocity point. For example, at the methanogenic half-velocity point, the equilibrium aqueous H_2 level, calculated from a headspace measurement, was on average more than three times the actual aqueous H_2 level. At the half-velocity point for dechlorination, the difference was on average more than 5-fold.

Probably due to the mass-transfer limitations associated with H_2 measurements, the literature reports $K_s(H_2)$ values for methanogens over the wide range of from 8 (27) to 13 000 nM (28); with such a wide range, the results of these experiments are easily consistent. This variability could be attributed to differences in liquid boundary layer thicknesses in systems caused by different mixing regimes. In estimating the bulk solution concentration at which velocity is half-maximum, a thicker boundary layer around the cells, caused by a lower mixing rate, would result in a higher value for the resulting $K_s(H_2)$ than in a system with a higher mixing rate and hence a thinner cell boundary layer. Different species of methanogens could exhibit different degrees of aggregation in suspended cultures that could also affect the H_2 transport from the bulk solution to the cell surface and then through the membrane. It is therefore important to examine the experimental method that investigators employed to measure these $K_s(H_2)$ values for methanogens before making a simple comparison. Also, it is crucial that, in comparing $K_s(H_2)$ values for dechlorinators versus methanogens, the mixing regime and/or bottle treatment be as uniform as possible among and between bottle sets. Because most of the results herein are based on comparisons between half-velocity constants for both processes when both were occurring in the same bottle,

this complication is avoided altogether; it becomes a problem only when comparing these values to those found in the literature.

The wide range in reported methanogenic $K_s(H_2)$ could also, in part, be a manifestation of an active formate/ H_2 lyase system in some methanogenic populations. Apparent values of $K_s(H_2)$ would be expected to vary with the ratio of formate/ H_2 at the half-velocity point. In fact, we observed that the methanogenic $K_s(H_2)$ was lower for formate-fed systems than H_2 -fed systems, presumably due to effects on the formate/ H_2 ratio and the resulting extracellular/intracellular H_2 gradient. In formate-fed systems, the intracellular H_2 concentration of organisms possessing the lyase system may become higher than in the bulk solution, whereas in H_2 -fed systems, the gradient is reversed. Additionally, the presence of the formate/ H_2 lyase system in a methanogen could contribute to variability among literature values of its $K_s(H_2)$ due to the dependence of the aqueous formate/ H_2 ratio on the bicarbonate concentration.

Since no studies in the literature have yet attempted to measure the $K_s(H_2)$ for a dechlorinating microbe, it is only possible to judge the validity of our data by their consistency with relative thermodynamic H_2 thresholds of dechlorinators and methanogens—i.e., the H_2 concentrations below which the free energies of the respective reactions cease to be negative. Free-energy calculations indicate a substantial difference—24 orders of magnitude—between the thermodynamic H_2 thresholds for the dechlorination of PCE to VC and for methanogenesis (21). Although the physiological thresholds can differ significantly from these values (due to various biochemical and microbiological factors), this difference in thermodynamic H_2 thresholds is so large that it might still be expected to manifest itself to some degree physiologically.

We are not able to provide accurate estimates of physiological H_2 thresholds for methanogens and dechlorinators from the experimental studies reported here—we employed dynamic, mixed-culture systems. H_2 consumption rates were never allowed to go to zero. Threshold estimates are best obtained in pure culture studies, where the H_2 can be allowed to drop to and rest at the physiological threshold, eliminating the need for mass-transfer corrections. The most that we can say at this time is that the physiological H_2 threshold for the PCE dechlorinators in our mixed culture is less than 2 nM—a quasi-steady-state concentration that sustained low rates of PCE dechlorination in propionate-fed bottles (29). This may be compared with compiled reports of physiological H_2 thresholds for methanogens which range from 23 to 75 nM (30), qualitatively supporting thermodynamic arguments that predict a much lower H_2 threshold for PCE dechlorinators than for methanogens.

While thresholds are not half-velocity constants, one might reasonably expect an organism with a low threshold to have evolved enzymatic machinery capable of handling H_2 efficiently at lower levels. This phenomenon has been investigated with regard to the analogous competition for H_2 between sulfate reducers and methanogens. Hydrogenotrophic sulfate reduction releases more energy than available from hydrogenotrophic methanogenesis. Thus, the thermodynamic H_2 threshold for sulfate reducers (ca. 10^{-7} atm) is approximately an order of magnitude lower than that of methanogens (ca. 10^{-6} atm) (31). As expected, this difference is reflected in their physiological H_2 thresholds (ca. 7 and 50 nM, respectively) (30); but it is also

reflected in their $K_s(H_2)$ values (ca. 2 and 8 μM , respectively) (28, 32). As a result, sulfate-reducers can out-compete methanogens at lower H_2 levels and eventually reduce the H_2 concentration to a level low enough to shut down methanogenic activity (28, 32, 33).

Because methanogens have been found to coexist with sulfate reducers even under sulfate-rich environments, Robinson and Tiedje (28) conjectured that it was perhaps the comparative ability of each organism to metabolize noncompetitive substrates that could also influence the populations and presence of the methanogens. Lovley et al. (33) however attributed the ability of methanogens to coexist with sulfate reducers to be a function of not only the relative $K_s(H_2)$ values but also the relative rates of H_2 production, the population sizes, and sulfate availability. In that study, the investigators predicted that the rate of H_2 production in Wintergreen Lake sediments was higher than the maximum estimate of the V_{max} of H_2 uptake by sulfate reducers. They predicted that in such sediments, sulfate availability would limit the maximum rate of H_2 utilization by sulfate reducers, allow the H_2 level to increase, and thus foster the coexistence of methanogens. This issue of availability could perhaps be a problem for dechlorinators under *in situ* conditions, with respect to PCE, in their competition for H_2 against methanogens. So long as PCE were available and the dechlorinators could maintain a low level of H_2 , however, their chances of outcompeting the methanogens for H_2 would be most improved. Further research, including field studies, could shed some light on how these results manifest themselves in the environment by investigating the electron donor or combination of donors that would take advantage of the dechlorinator's ability to outcompete methanogens for H_2 at very low levels.

Acknowledgments

This research was supported by the U.S. Air Force Armstrong Laboratory, Environmental Quality Directorate, Tyndall AFB, FL, under Contract F08635-93-C-0077, and through a Graduate Research Fellowship from the National Science Foundation. The authors gratefully acknowledge the contribution of Donna Fennell, School of Civil & Environmental Engineering, Cornell University, who developed and maintained the PCE/butyrate enrichment culture.

Literature Cited

- (1) deBruin, W. P.; Kotterman, M. J. J.; Posthumus, M. A.; Schraa, G.; Zehnder, A. J. B. *Appl. Environ. Microbiol.* **1992**, *58*, 1996–2000.
- (2) Freedman, D. L.; Gossett, J. M. *Appl. Environ. Microbiol.* **1989**, *55*, 2144–2151.
- (3) McCarty, P. L. *Curr. Opin. Biotechnol.* **1993**, *4*, 323–330.
- (4) Vogel, T. M.; Criddle, C. S.; McCarty, P. L. *Environ. Sci. Technol.* **1987**, *21*, 722–736.
- (5) DiStefano, T. D.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* **1991**, *57*, 2287–2292.
- (6) DiStefano, T. D.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* **1992**, *58*, 3622–3629.
- (7) Maymó-Gatell, X.; Tandoi, V.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* **1995**, *61*, 3928–3933.
- (8) Gibson, S. A.; Sewell, G. W. *Appl. Environ. Microbiol.* **1992**, *58*, 1392–1393.
- (9) Holliger, C.; Schraa, G.; Stams, A. J. M.; Zehnder, A. J. B. *Appl. Environ. Microbiol.* **1993**, *59*, 2991–2997.
- (10) Holliger, C.; Schumacher, W. *Antonie van Leeuwenhoek* **1994**, *66*, 239–246.
- (11) Neumann, A.; Scholz-Muramatsu, H.; Diekert, G. *Arch. Microbiol.* **1994**, *162*, 295–301.
- (12) Stover, M. A. M.S. Thesis, Cornell University, Ithaca, NY, 1993.
- (13) Fennell, D. E.; Stover, M. A.; Zinder, S. H.; Gossett, J. M. In *Bioremediation of Chlorinated Solvents*; Hincsee, R. E., Leeson, A., Semprini, L., Eds.; Battelle Press: Columbus, OH, 1995; pp 9–16.
- (14) DiStefano, T. D. Ph.D. Thesis, Cornell University, Ithaca, NY, 1992.
- (15) Zehnder, A. J. B.; Wuhrmann, K. *Science* **1976**, *194*, 1165–1166.
- (16) Balch, W. E.; Fox, G. E.; Magrum, L. J.; Woese, C. R.; Wolfe, R. S. *Microbiol. Rev.* **1979**, *43*, 260–296.
- (17) Pauss, A.; Andre, G.; Perrier, M.; Guiot, S. R. *Appl. Environ. Microbiol.* **1990**, *56*, 1636–1644.
- (18) Robinson, J. A.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1982**, *44*, 1374–1384.
- (19) Boone, D. R.; Johnson, R. L.; Liu, Y. *Appl. Environ. Microbiol.* **1989**, *55*, 1735–1741.
- (20) Schauer, N. L.; Ferry, J. G. *J. Bacteriol.* **1980**, *142*, 800–807.
- (21) Smatlak, C. R. M.S. Thesis, Cornell University, Ithaca, NY, 1995.
- (22) Young, C. L. *Hydrogen and Deuterium*; Pergamon Press: New York, 1981.
- (23) Tandoi, V.; DiStefano, T. D.; Bowser, P. A.; Gossett, J. M.; Zinder, S. H. *Environ. Sci. Technol.* **1994**, *28*, 973–979.
- (24) Perry, R. H.; Chilton, C. H. *Chemical Engineer's Handbook*, 5th ed.; McGraw-Hill: New York, 1973.
- (25) Reid, R. C.; Prausnitz, J. M.; Sherwood, T. K. *The Properties of Gases and Liquids*; McGraw-Hill: New York, 1977.
- (26) Maymó-Gatell, X.; Chien, Y.; Anguish, T.; Gossett, J.; Zinder, S. unpublished results, 1996.
- (27) Giraldo-Gomez, E.; Goodwin, S.; Switzenbaum, M. S. *Biotechnol. Bioeng.* **1992**, *40*, 768–776.
- (28) Robinson, J. A.; Tiedje, J. M. *Arch. Microbiol.* **1984**, *137*, 26–32.
- (29) Fennell, D. E.; Zinder, S. H.; Gossett, J. M. Cornell University, unpublished results, 1996.
- (30) Cord-Ruwisch, R.; Steitz, H.-J.; Conrad, R. *Arch. Microbiol.* **1988**, *149*, 350–357.
- (31) Zinder, S. H. In *Methanogenesis: Ecology, Physiology, Biochemistry & Genetics*; Ferry, J. G., Ed.; Chapman & Hall: New York, 1993; pp 128–206.
- (32) Kristjansson, J. K.; Schönheit, P.; Thauer, R. K. *Arch. Microbiol.* **1982**, *131*, 278–282.
- (33) Lovley, D. R.; Dwyer, D. F.; Klug, M. J. *Appl. Environ. Microbiol.* **1982**, *43*, 1373–1379.

Received for review March 15, 1996. Revised manuscript received May 17, 1996. Accepted May 17, 1996.*

ES9602455

* Abstract published in *Advance ACS Abstracts*, July 1, 1996.

Reductive Dehalogenation of Chlorinated Ethenes and Halogenated Ethanes by a High-Rate Anaerobic Enrichment Culture

Valter Tandoi,^{†‡} Thomas D. DiStefano,^{§,||} Patrick A. Bowser,[†] James M. Gossett,[§] and Stephen H. Zinder^{*†}

Section of Microbiology, Wing Hall, and School of Civil and Environmental Engineering, Hollister Hall, Cornell University, Ithaca, New York 14853

An anaerobic enrichment culture, using CH₃OH as an electron donor, dechlorinated tetrachloroethene (PCE, 55 μ mol added/100 mL of culture) nearly stoichiometrically to vinyl chloride (VC) in 20 h with negligible buildup of other intermediates and at a maximum rate of 4.6 ± 0.4 μ mol of PCE transformed/mg of volatile suspended solids per day. Appreciable conversion of VC to ETH occurred only after the PCE was nearly depleted, suggesting the inhibition of VC dechlorination by PCE. PCE, trichloroethene, *cis*-1,2-dichloroethene (DCE), and 1,1-DCE were all rapidly metabolized to VC with near zero-order kinetics and apparently inhibited subsequent VC dechlorination. *trans*-1,2-DCE was converted to VC with first-order kinetics and did not inhibit VC dechlorination. VC, when added alone, was dechlorinated to ETH with no lag and with first-order kinetics (half-life = 17.3 h). A computer simulation of the experimental data for PCE conversion to ETH was obtained using kinetic parameters estimated for the individual chlorinated ethenes and assuming competitive inhibition of VC dechlorination by the other chlorinated ethenes. The rates of PCE dechlorination by this culture were significantly higher than those found for cometabolic PCE transformation by anaerobes, and the kinetics are considerably different from those obtained for reductive dechlorination *in vitro* by transition metal cofactors. The culture rapidly dehalogenated 1,2-dichloroethane or 1,2-dibromoethane to ETH, but did not use trichloroethanes, tetrachloromethane, trichloromethane, or dichloromethane.

Introduction

Chlorinated ethenes are widely used solvents and chemical feedstocks. They are considered to be major groundwater contaminants (1, 2), and considerable effort has been expended in understanding and improving microbial degradation of these compounds. Trichloroethene (TCE) can be cometabolized by a variety of oxygenase-containing aerobic microorganisms (3), while tetrachloroethene (PCE) is not known to be metabolized or cometabolized by aerobes. However, it is now well established that PCE can be reductively dechlorinated by microorganisms in anaerobic habitats to trichloroethene (TCE), dichloroethene (DCE) isomers, and vinyl chloride (VC) (4–9). Complete dechlorination of PCE to ethene (ETH) (10, 11) or ethane (12) has been reported. It is not clear why some anaerobic systems only partially dechlorinate PCE, while others effect complete dechlorination.

Little is known about the organisms involved in reductive dechlorination of chlorinated ethenes in natural

systems. Pure cultures of *Methanosarcina* and *Desulfomonile tiedjei* have been shown to carry out reductive dechlorination of PCE to TCE (13–16). These transformations are apparently cometabolic since they occur at relatively low rates and there is no evidence for benefit to the organism. Recently, a highly purified anaerobic culture was described which grew by reductively dechlorinating PCE to *cis*-DCE using H₂ as an electron donor (17).

A plausible mechanism for reductive dechlorination of PCE by methanogens and other anaerobes is mediation by reduced transition metal cofactors. Gantzer and Wackett (18) examined dechlorination catalyzed by vitamin B₁₂ and cofactor F₄₃₀ using Ti³⁺ as the reductant. Vitamin B₁₂ and related corrinoids are abundant in methanogens (19) and other anaerobes, especially acetogens (20). Coenzyme F₄₃₀ is a prosthetic group in the "methyl reductase" enzyme complex in methanogens (19). Both of these cofactors were capable of reductive dechlorination of chlorinated ethenes using the strong reductant Ti³⁺ as the electron donor. In the sequential reduction of PCE to ETH, each step was first-order, and each succeeding step was over 10-fold slower than that preceding it, so that VC reduction to ETH was ca. 10 000-fold slower than PCE reduction to TCE at equivalent concentrations using either F₄₃₀ or vitamin B₁₂ as a catalyst. Similar results have been obtained for carbon monoxide dehydrogenase (21), a corrinoid-containing enzyme found in methanogens and acetogens. Transition metal cofactors can catalyze many other reductive dehalogenations including dihalo elimination from 1,2-dibromoethane (22) and 1,2-dichloroethane (23), forming ETH. Trichloroethanes can also be dehalogenated by transition metal cofactors, but at a relatively low rate (24). Thus, anaerobes that are rich in reduced transition metal cofactors, most notably methanogens and acetogens, may catalyze reductive dechlorination in anaerobic habitats, but at low rates, especially for less chlorinated species.

We have recently described an anaerobic enrichment culture that rapidly dechlorinates high concentrations of PCE [550 μ mol added/L, ca. 322 μ mol/L of aqueous concentration (25)] to ETH using CH₃OH as an electron donor (10). In that study, it was shown that once the culture was adapted to high PCE concentrations, over 80% of the added PCE was converted to ETH within 2 days, while VC accounted for essentially all the remainder of the PCE metabolites. For cultures incubated 4 days, ETH represented over 99% of the products. These results indicate that VC dechlorination to ETH was the rate-limiting step for reductive dechlorination by the culture. More recently, we have presented evidence that H₂ is the actual electron donor for reductive dechlorination by the methanol/PCE culture (26). In this study, we examine the kinetics of PCE conversion to ETH more closely and examine the ability of the culture to dechlorinate other chlorinated ethenes and some halogenated ethanes and ethenes.

* Corresponding author: Section of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853; Telephone: (607)255-2415; FAX: (607)255-3904.

[†] Section of Microbiology.

[‡] Present address: CNR IRSA, via Reno 1, 00198 Rome, Italy.

[§] School of Civil and Environmental Engineering.

^{||} Present address: Pennsylvania State University—Harrisburg, Middletown, PA 17057.

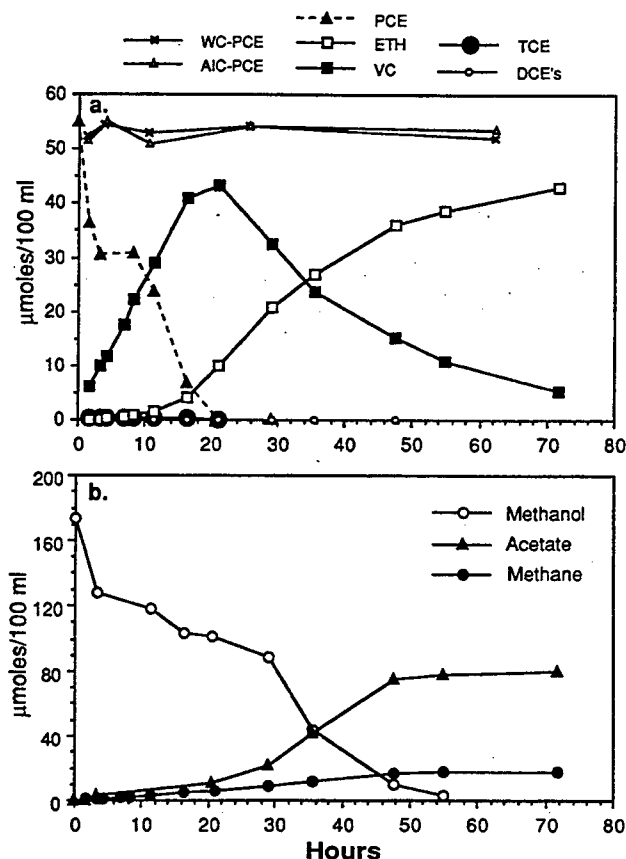


Figure 1. Kinetics of (a) PCE transformation and (b) methanol metabolism by the $\text{CH}_3\text{OH}/\text{PCE}$ enrichment culture receiving a standard dose of PCE ($55\text{ }\mu\text{mol}/100\text{ mL}$) and CH_3OH ($160\text{ }\mu\text{mol}/100\text{ mL}$). The acetate measurements represent the net increase from the concentration at zero time. WC, water control; AIC, autoclaved inoculated control (see Materials and Methods).

Materials and Methods

Growth Media and Culture Conditions. A 6-L $\text{CH}_3\text{OH}/\text{PCE}$ enrichment culture was maintained at 35°C as an inoculum source for the study on the kinetics of PCE and CH_3OH utilization (Figure 1). This source reactor was a stirred, 9.6-L bottle containing 6 L of basal medium (11), sealed by a Teflon-lined rubber gasket and a stainless-steel plate, and was provided with substrate every 2 days (retention time = 20 days) as has been previously described (26).

In subsequent studies, the culture was adapted to a basal growth medium which does not contain iron sulfide and other precipitates (27) and is designated "clear medium". This medium was dispensed inside an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) in 100-mL amounts into 160-mL serum bottles, which were then sealed with Teflon-coated butyl rubber stoppers (Wheaton, Millville, NJ) that were previously autoclaved to reduce the emission of volatile organic compounds. The bottle headspaces were flushed with a 70% N_2 -30% CO_2 mixture (Matheson Gas Products, East Rutherford, NJ) and received the following additions (per 100 mL of medium) before inoculation: 1 mL of vitamin solution (28), 0.5 mL of 5% w/v $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$, 2 mL of 10% w/v NaHCO_3 , and 0.1 mL of 10% w/v yeast extract. The initial amounts of substrates added were 20 μmol of PCE and 64 μmol of CH_3OH , and subsequent feedings were 55 μmol of PCE and 160 μmol of CH_3OH . Once established, these cultures

performed identically with those in the original medium. Sodium bicarbonate was periodically added to neutralize the hydrochloric and acetic acids produced by the dechlorination and acetogenesis. Methanogenesis in these cultures was variable, but always represented less than 10% of the total electron flow. From one of these cultures, a culture was established in 1.5 L of clear medium inside a 2.3-L bottle, the opening of which was modified to accept a 20-mm serum stopper. This culture was used in kinetic experiments involving chlorinated ethenes and ethanes (Figures 2-5). This culture was operated in essentially a batch mode except that the medium removed for experiments was replaced with fresh reduced basal medium.

PCE and Methanol Utilization Experiments. A 100-mL sample from the 6-L $\text{CH}_3\text{OH}/\text{PCE}$ culture described above was anaerobically dispensed into each of five 160-mL serum bottles using a Unispense II pump. The culture had been purged with 70% N_2 -30% CO_2 to remove chlorinated and other volatile organic compounds. The bottles were sealed with Teflon-lined butyl rubber stoppers. Three replicates were designated to study the rates of PCE transformation: each received 55 μmol of PCE, 160 μmol of methanol, and 2 mg of yeast extract. Two bottles were designated as autoclaved inoculated controls (AIC) to determine the potential abiotic transformation of PCE, were autoclaved for 1 h at 130°C , and were cooled to 35°C prior to the addition of PCE and CH_3OH . Two additional serum bottles received 100 mL of distilled water and PCE and were designated water controls (WC). All bottles were incubated upside down in an orbital shaker bath at 35°C . Biomass was measured as volatile suspended solids (29). Results are presented for individual bottles, but replicate bottles performed similarly with respect to patterns of PCE dechlorination, rates of product formation, and methanol utilization.

Utilization of Chlorinated Ethenes, Ethanes, and Methanes. These experiments used culture adapted to clear medium, as described above. The 1.5-L culture was sparged with 70% N_2 -30% CO_2 to remove volatiles, and 25-mL samples were added inside an anaerobic glovebox to each of the 12 36-mL serum bottles. The bottles were sealed with Teflon-lined butyl rubber stoppers and were flushed with 70% N_2 -30% CO_2 . Chlorinated ethenes were added neat using a 10- μL microsyringe except for VC, which was added as a gas. Each bottle received 11.25-13.75 μmoles of a chlorinated ethene, equivalent to 45-55 $\mu\text{mol}/100\text{ mL}$. The bottles also received the equivalent of 340 $\mu\text{mol}/100\text{ mL}$ of CH_3OH , more than a 4-fold excess in terms of electron equivalents, to ensure that methanol was not depleted during the time course. This was verified by detecting residual methanol, using an alcohol oxidase assay (30) in bottles at the end of the incubation period. The bottles also received 1 mg/100 mL of yeast extract. The bottles were incubated upside down in a 35°C shaking water bath. Since the medium did not contain particulate matter, biomass was measured as dry weight by filtering 10-mL samples through 0.2- μm Nucleopore membrane filters (Fisher Scientific, Rochester, NY) and measuring the weight increase. The results are presented for individual bottles, but duplicates performed similarly, and the experiments presented were repeated at least once with similar results.

Analytical Techniques. In the experiment reported in Figure 1, volatile components were measured in the culture headspaces using the tandem flame ionization

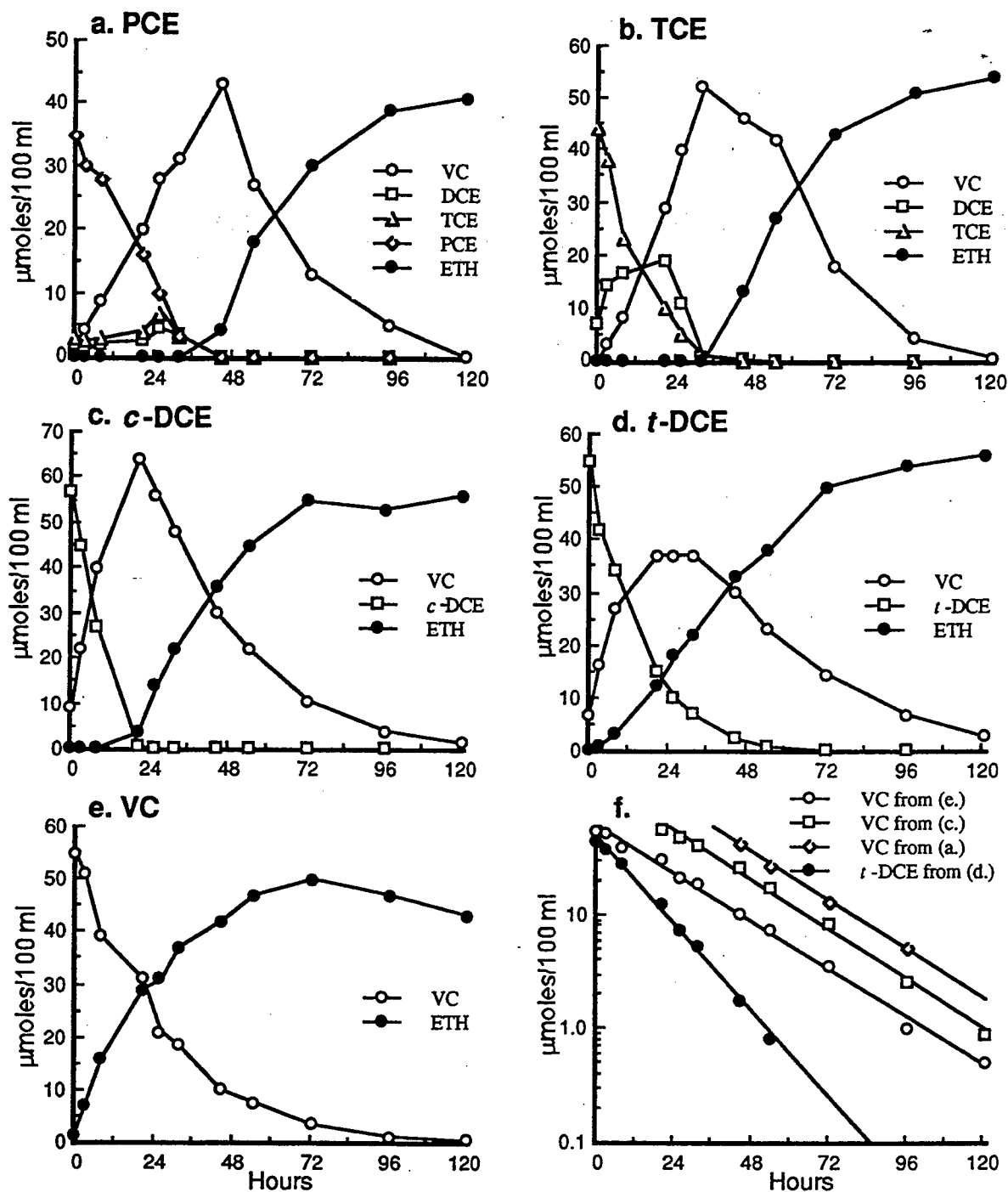


Figure 2. Conversion of chlorinated ethenes to ethene by a $\text{CH}_3\text{OH}/\text{PCE}$ enrichment culture adapted to growth in clear medium. Individual serum vials received 45–55 $\mu\text{mol}/100\text{ mL}$ of a chlorinated ethene and an excess amount of CH_3OH (340 $\mu\text{mol}/100\text{ mL}$, see Materials and Methods). (a) PCE; (b) TCE; (c) *cis*-DCE; (d) *trans*-DCE; (e) VC; (f) semilogarithmic plot for disappearances of VC in Figure 2e (VC alone), Figure 2c (*cis*-DCE), or Figure 2a (PCE), and for the disappearance of *trans*-DCE in Figure 2d.

detector gas chromatographic system previously described (10). Acetate and methanol were analyzed using a Perkin Elmer 8500 flame ionization detector gas chromatograph. A 0.53 mm \times 15 m Nukol fused-silica capillary column (Supelco, Inc., Bellefonte, PA) was used, and the oven temperature was 100 $^{\circ}\text{C}$ for three minutes followed by a 20 $^{\circ}\text{C}/\text{min}$ increase to 160 $^{\circ}\text{C}$. Samples (100–200 μL) were filtered through a 0.45- μm syringe filter (Gelman Sciences, Ann Arbor, MI) and were acidified with 5 μL of 2 N HCl, and a 0.5- μL sample was injected. Peak areas responses for methanol and acetate were compared to standard curves.

In subsequent experiments, VC, DCE isomers, TCE, PCE, 1,2-dibromoethane, 1,2-dichloroethane, 1,1,1-trichloroethane, and chloromethanes were quantified using a Varian 1400 flame ionization detector gas chromatograph with a 2 m \times 3 mm stainless steel column packed with 60/80 mesh Carbowax B/1% SP-1000 (Supelco) and operated isothermally at 210 $^{\circ}\text{C}$. Headspace samples were 100- μL and were taken using a 250- μL Pressure Loksyringe (Supelco). CH_4 , ETH, and ethane were quantified in separate 100- μL headspace samples using a Varian 1400 flame ionization detector gas chromatograph outfitted with 2 m \times 3 mm Poropak R column (Supelco) operated at 40

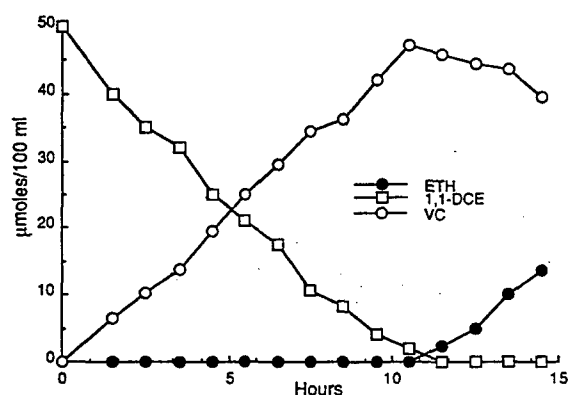


Figure 3. Conversion of 1,1-DCE to ethene by a $\text{CH}_3\text{OH}/\text{PCE}$ enrichment culture adapted to clear medium. Conditions were as in Figure 2, except that a parallel culture-fed PCE completely converted the PCE to VC within 24 h.

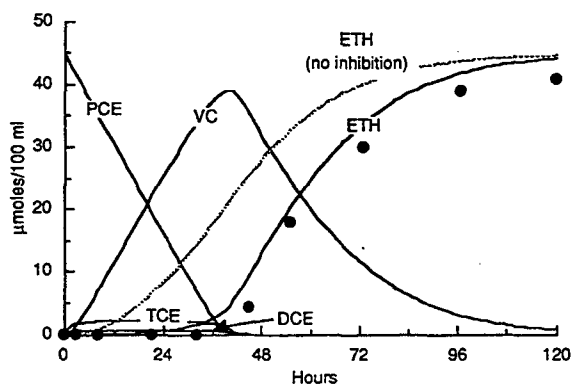


Figure 4. Simulation of PCE conversion to ETH using parameters derived from the results in Figure 2 and assuming that the initial PCE dose was $45 \mu\text{mol}/100 \text{ mL}$ of culture, that *cis*-DCE was the primary intermediate, and that there was competitive inhibition of VC dechlorination to ETH by other chlorinated ethenes (see text). Also included is a simulation of ETH production if no inhibition of VC dechlorination is assumed and the actual ETH accumulation data from Figure 2a (●).

°C. The carrier gas was N_2 with a flow of $30 \text{ mL}/\text{min}$ in both instruments. Standards were prepared by adding a known amount of the compound to a bottle with the same headspace to liquid ratio as the samples being analyzed (25). It was assumed that DCE produced from more highly chlorinated ethenes was *cis*-DCE, which had a somewhat different calibration constant from *trans*-DCE because of differences in their Henry's constants (25). CH_3OH was quantified in these studies by an enzymatic assay using alcohol oxidase (30).

Chemicals. PCE, TCE, *cis*-1,2-DCE, *trans*-1,2-DCE, 1,1-DCE, 1,2-dibromoethane, 1,2-dichloroethane, 1,1,1-trichloroethane, and 1,1,2-trichloroethane were purchased from Aldrich Chemical, Co. (Milwaukee, WI). VC was obtained as a gas from Matheson. Tetrachloromethane, trichloromethane, and dichloromethane were from Fisher Scientific. Alcohol oxidase and other test reagents for the CH_3OH assay were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

PCE and Methanol Utilization. Figure 1a shows a time course for metabolism of the standard dose (10) of $55 \mu\text{mol}$ of PCE/100 mL and $160 \mu\text{mol}$ of $\text{CH}_3\text{OH}/100 \text{ mL}$ by a $\text{CH}_3\text{OH}/\text{PCE}$ culture. PCE was undetectable within 20 h, and there was a concomitant increase in VC. The

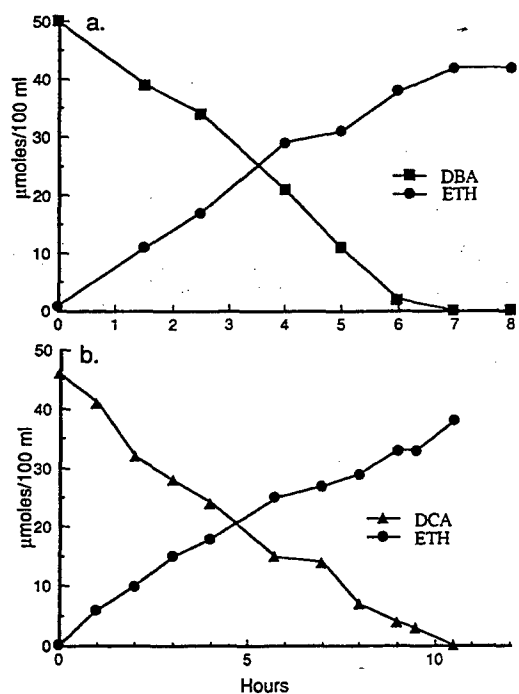


Figure 5. Conversion of (a) 1,2-dibromoethane (DBA) and (b) 1,2-dichloroethane (DCA) to ETH by the $\text{CH}_3\text{OH}/\text{PCE}$ enrichment culture adapted to clear medium. Conditions were as in Figure 2, except that a parallel culture-fed PCE completely converted the PCE to VC within 12 h.

smoothness of the increase in VC suggests that the biphasic nature of the PCE decrease is artifactual, most likely due to incomplete dissolution of PCE, which was at concentrations close to saturation. The time-zero value for PCE in Figure 1a was calculated—not measured. It was based on known PCE addition and probably depicts accurately the total micromoles of PCE in the bottle. The subsequent two points were measured values which likely underestimated the total PCE remaining, due to the presence of undissolved PCE. At 16 h, when the VC concentration was high and the PCE was nearly completely consumed, ETH accumulation was detected. Thereafter, VC declined to $5 \mu\text{mol}/100 \text{ mL}$ and ETH accumulated to $43 \mu\text{mol}/100 \text{ mL}$. TCE and DCE isomers remained at insignificant levels during the experiment. There was essentially no PCE disappearance in autoclaved cultures or in water controls, indicating that abiotic PCE transformation and sorption of added PCE were insignificant. The culture biomass was determined as 127 mg of volatile suspended solids/L, and sustained rates of PCE dechlorination to VC for three replicate bottles averaged $4.6 \pm 0.4 \mu\text{mol}$ ($0.76 \pm 0.07 \text{ mg}$) of PCE transformed/mg of volatile suspended solids per day.

Figure 1b shows that after an initial rapid decrease, methanol utilization was slow until 30 h, after which time the rate increased, associated with increased methanogenesis and acetogenesis. Methanol was depleted by 55 h and could not have contributed electrons to subsequent VC dechlorination. On a reducing equivalent basis, the amount of reduced products (ETH, VC, acetate, and CH_4) formed exceeded the amount of electrons potentially donated by the methanol by approximately 10%, most likely due to reducing power provided by the yeast extract added as has been found previously (10).

Utilization of Chlorinated Ethenes. The ability of a culture adapted to clear medium (see Materials and

Methods) to use a variety of chlorinated ethenes was examined. The culture required 36–46 h to completely degrade PCE (Figure 2a), and there was some accumulation of TCE and DCEs, possibly due to the perturbation of the cultures during manipulation since accumulation of these intermediates was not detected in the source culture (data not presented). As in Figure 1, there was probably incomplete equilibration of PCE during early time points, and the measured values are underestimates. There was essentially stoichiometric conversion of PCE in this culture to VC, and VC dechlorination to ETH occurred only when the other chlorinated ethenes were nearly completely consumed. A maximum specific rate for PCE conversion to VC of $2.7 \mu\text{mol}$ (0.45 mg) of PCE/mg of dry weight per day was estimated for this culture.

TCE was dechlorinated at rates comparable to PCE (Figure 2b), and there was some DCE buildup. VC conversion to ETH was found only after more chlorinated ethenes were absent. *cis*-DCE was completely dechlorinated to VC within 20 h (Figure 2c), and similar to PCE and TCE, ETH was only detected after *cis*-DCE was consumed. *trans*-DCE was degraded more slowly than was *cis*-DCE (Figure 2d), and a semilogarithmic plot of *trans*-DCE disappearance (Figure 2f) yielded a straight line ($r = 0.9979$), indicating first-order kinetics and an estimated half-life of 9.5 h. Moreover, there was significant ETH appearance well before complete disappearance of *trans*-DCE, and the maximum VC accumulation was considerably less than for PCE, TCE, or *cis*-DCE.

VC, when added alone, was converted to ETH by the culture with little or no lag (Figure 2e). A semilogarithmic plot of VC disappearance (Figure 2f) was linear ($r = 0.9974$), indicating first-order disappearance, with an estimated half-life of 17.3 h. Also plotted in Figure 2f are the disappearances of VC in the bottle fed *cis*-DCE (from Figure 2c) and the bottle-fed PCE (from Figure 2a). They also formed straight lines with slopes similar to that for VC alone (16.5 and 16.8 h half-lives respectively), indicating that once the more chlorinated ethenes had disappeared, the kinetics of VC dechlorination were similar to those of the bottle receiving VC alone. The estimated half-life for VC in the *trans*-DCE culture was 22 h (data not shown). The disappearance of ETH from the bottle-fed VC at 96 and 120 h could not be accounted for as either ethane or methane and may have represented leakage or analytical error.

Utilization of 1,1-DCE by the culture was examined in a separate experiment. As shown in Figure 3, it was rapidly converted to VC, and VC conversion to ETH only occurred after 1,1-DCE was consumed.

Simulation of Chlorinated Ethene Utilization. The results in Figure 2 suggested that PCE, TCE, and *cis*-DCE disappearances followed zero-order kinetics in the concentration range measured, i.e., having an apparent K_m value lower than most or all of the data points measured, while VC (and *trans*-DCE) followed first-order kinetics in that range. This interpretation was examined further by using Stella II simulation software for the Macintosh computer (High Performance Systems, Hanover, NH). It was assumed that biomass did not increase during the incubation period since the retention time for the culture studied in Figure 1 was 20 days, with a feeding every 2 days so that the biomass would only increase ca. 10% after one feeding. The batch-fed culture used in Figure 2 had an even longer retention time. Michaelis-

Menten parameters were assigned by visually comparing the disappearance of each chlorinated ethene in Figure 2 with simulations. If a reaction follows zero-order kinetics, a V_{\max} value is known, but the K_m value, if it exists, is lower than most or all of the points measured. For PCE disappearance in Figure 2a, except for the early time points which are considered underestimates (see above), there was a good fit assuming a V_{\max} value of $1.25 \mu\text{mol/h}$ for 100 mL of culture and an arbitrary K_m value of $1 \mu\text{mol}/100 \text{ mL}$ of culture [ca. $0.6 \mu\text{M}$ of dissolved PCE (25)]. By similar methods, V_{\max} values of 4.0 and $3.0 \mu\text{mol/h}$ for 100 mL of culture were estimated and K_m values of 5 and $1 \mu\text{mol}/100 \text{ mL}$ of culture were chosen for TCE and *cis*-DCE, respectively. Since VC and *trans*-DCE utilizations exhibited first-order kinetics, one can obtain the ratio of V_{\max}/K_m from the data, which were approximately 0.06 and 0.1 h^{-1} , respectively. Using these ratios, an arbitrary K_m value of $40 \mu\text{mol}/100 \text{ mL}$ would give first-order kinetics over most of the range studied and yield V_{\max} values of 4.5 and $2.5 \mu\text{mol/h}$ for 100 mL of culture, respectively.

Using these parameters for the individual chlorinated ethenes, the stepwise dechlorination of PCE to ETH was simulated. The primary assumptions in constructing the model were that *cis*-DCE was the primary DCE isomer serving as an intermediate in PCE dechlorination to ETH and that there was competitive inhibition of VC conversion to ETH by the other chlorinated ethenes with $K_i = 1 \mu\text{mol}/100 \text{ mL}$, the arbitrary K_m value for PCE and *cis*-DCE dechlorination. Competitive and noncompetitive inhibition are indistinguishable in first-order kinetics, i.e., at substrate concentrations well below the K_m value. As shown in Figure 4, these assumptions gave a simulation qualitatively similar to the results shown in Figure 2a. If it was assumed that *trans*-DCE was the primary intermediate in PCE dechlorination, the simulation predicted much greater DCE persistence than was found (simulation not shown). The predicted ETH formation in the simulation was slightly higher than the measured accumulation. However, if no inhibition of VC dechlorination was assumed, then the model predicted much earlier accumulation of ETH than was observed (Figure 4). There would also be correspondingly less VC accumulation (simulation not shown).

Metabolism of Halogenated Ethanes and Methanes. The culture rapidly converted high concentrations of 1,2-dibromoethane or 1,2-dichloroethane to ETH (Figure 5). No ethane or acetylene was detected, and there was a relatively small peak which may have represented chloroethane (23) in samples from 1,2-dichloroethane. 1,1,1-Trichloroethane and 1,1,2-trichloroethane, tetrachloromethane, trichloromethane, and dichloromethane were not significantly metabolized by the culture after 24-h incubation.

Discussion

The high-rate $\text{CH}_3\text{OH}/\text{PCE}$ anaerobic enrichment culture was capable of reductive dechlorination of all chlorinated ethenes, consistent with sequential reductive dechlorination of PCE to ETH. The pattern of dechlorination is considerably different from that catalyzed in vitro by reduced transition metal cofactors (18). While VC dechlorination to ETH was the rate-limiting step in the culture, the rates of dechlorination of all the chlorinated ethenes, including VC when PCE was not present, were

comparable to that for PCE rather than being orders of magnitude lower as in the case of dechlorination by vitamin B₁₂ and cofactor F₄₃₀. The only chlorinated ethenes showing first-order dechlorination kinetics were VC and *trans*-DCE. The near zero-order kinetics found for the dechlorination of high concentrations of the other chlorinated ethenes is suggestive of mediation by enzymes with K_m values which are low relative to the concentration range in which most of our measurements were made (5–50 $\mu\text{mol}/100\text{ mL}$).

The CH₃OH/PCE culture dechlorinated PCE to VC at extremely high rates. For comparison, in a survey of pure cultures of anaerobes (13), the two highest rates of conversion of PCE to TCE, a two-electron reduction, were 2.34 and 0.84 nmol/mg of protein each day for *D. tiedjei* and *Methanosarcina barkeri*, respectively. These rates are over 3 orders of magnitude lower than rates of PCE reduced to VC, a 6-electron reduction, for the CH₃OH/PCE culture: 2.7–4.6 μmol of PCE dechlorinated to VC/mg of volatile suspended solids (or dry weight) each day.

Thus, the kinetics of dechlorination do not resemble those for catalysis by transition metal cofactors, and the rates of dechlorination are considerably higher than those for cultures carrying out cometabolic dechlorination. These factors, plus our ability to transfer the PCE dechlorination activity (26), argue that organisms in the culture may be deriving benefit from PCE dechlorination, perhaps as an electron acceptor for energy conservation using H₂ as the donor (26). The E' values for reductive dechlorination of chlorinated ethenes range from +0.36 to +0.58 V (31), close to the NO₃⁻/NO₂⁻ couple (+0.42 V). This proposition is also bolstered by the recent demonstration of growth of a highly purified culture on H₂ and PCE producing *cis*-DCE (17).

Estimates for the kinetic parameters for each of the chlorinated ethenes gave a realistic simulation of the sequential dechlorination of PCE to ETH (Figure 4). It should be emphasized that the estimated K_m values used were arbitrary, since they were either lower than most of the data points (PCE, TCE, *cis*-DCE, and 1,1-DCE) or were greater than them (*trans*-DCE and VC). We are presently performing more detailed studies on the kinetics of chloroethene dechlorination by the culture.

One assumption of the model was that *cis*-DCE was the isomer serving as the major intermediate in PCE dechlorination. Our analytical techniques did not separate DCE isomers, but *cis*-DCE has been found to be the major intermediate in reductive dechlorination of chlorinated ethenes in other systems (12, 32), although *trans*-DCE has been detected (33) including in a culture from which the presently described culture was derived (11). The kinetics of 1,1-DCE dechlorination are such that it could also be an intermediate in PCE dechlorination by this culture. 1,1-DCE has been reported to form from the abiotic reaction of PCE or TCE with sulfide (32).

The other major assumption of the model was that VC dechlorination was inhibited by PCE and other chlorinated ethenes. There was clear inhibition of VC dechlorination by all other chlorinated ethenes except *trans*-DCE. For simplicity, we assumed competitive inhibition with $K_i = K_m$ for PCE, which gave a realistic simulation; however, we have no evidence that the inhibition was actually competitive, and noncompetitive inhibition is indistinguishable from competitive inhibition when $S \ll K_m$. This inhibition phenomenon may be relevant to PCE or TCE

dechlorination when at high concentrations in anaerobic natural systems. It should also be emphasized that while the inhibition of VC transformation by other chlorinated ethenes was always found, the degree of inhibition was variable (compare Figures 1 and 2a), and that the kinetic parameters used in the simulation in Figure 4 are estimates and would not predict the behavior of chlorinated ethenes at low concentrations. Inhibition of reductive dechlorination of a less chlorinated intermediate by a more chlorinated precursor is not unprecedented. For example, Suflita et al. (34) found that 3-chlorobenzoate accumulated as an intermediate during 3,5-dichlorobenzoate dechlorination by a mixed anaerobic enrichment culture. 3-Chlorobenzoate was only utilized when 3,5-dichlorobenzoate was depleted, and the kinetics of inhibition fit several models, including competitive inhibition.

VC dechlorination to ETH leads to essentially complete detoxification of the chlorinated ethenes and is, therefore, a critical step. VC dechlorination showed first-order kinetics, so that its disappearance was concentration-dependent. First-order dechlorination kinetics and inhibition by other chlorinated ethenes may limit VC dechlorination in bioreactors or in situ if this pattern holds true for other systems. It should be mentioned, however, that VC is more readily degraded than TCE in aerobic systems (35) so that sequential anaerobic/aerobic systems (36) may be feasible, even if anaerobic biodegradation does not lead to complete conversion to ETH.

The CH₃OH/PCE culture rapidly carried out rapid dihalo elimination to ethylene of either 1,2-dichloroethane, a common solvent, and 1,2-dibromoethane, a now-banned fumigant. These eliminations are carried out by methanogenic cultures (23, 37, 38) and by corrinoids or cofactor F₄₃₀ (22, 23, 31). Interestingly, 1,1,2-trichloroethane, which only differs from 1,2-dichloroethane by a single chlorine, was not rapidly dechlorinated by the CH₃OH/PCE culture and neither was 1,1,1-trichloroethane, a common solvent, nor were chlorinated methanes. Thus, the culture shows considerable specificity in its ability to dehalogenate halo aliphatic compounds.

Acknowledgments

We are grateful to Rob Sandoli, who performed preliminary versions of some of the experiments presented here. This research was supported by the United States Air Force's Armstrong Laboratory, Environmental Quality Directorate, Tyndall Air Force Base, Florida, and by the Cornell Center for Advanced Technology (CAT) in Biotechnology, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the National Science Foundation. V.T. was supported by NATO Senior Fellowship 217.24.16.

Literature Cited

- (1) *Fed. Reg.* 1989, 54, 22062–22160.
- (2) Westrick, J. J.; Mello, J. W.; Thomas, R. F. *J. Am. Water Works Assoc.* 1984, 76 (5), 52–59.
- (3) Ensley, B. D. *Annu. Rev. Microbiol.* 1991, 45, 283–299.
- (4) Barrio-Lage, G.; Parsons, F. Z.; Nassar, R. S. *Environ. Sci. Technol.* 1987, 21, 366–370.
- (5) Bouwer, E. J.; McCarty, P. L. *Appl. Environ. Microbiol.* 1983, 45, 1286–1294.
- (6) Bagley, D. M.; Gossett, J. M. *Appl. Environ. Microbiol.* 1990, 56, 2511–2516.
- (7) Gibson, S. A.; Sewell, G. W. *Appl. Environ. Microbiol.* 1992, 58, 1392–1393.

- (8) Parsons, F.; Wood, P. R.; DeMarco, J. J. *Am. Water Works Assoc.* 1984, 76 (2), 56-59.
- (9) Vogel, T. M.; McCarty, P. L. *Appl. Environ. Microbiol.* 1985, 49, 1080-1083.
- (10) DiStefano, T. D.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* 1991, 57, 2287-2292.
- (11) Freedman, D. L.; Gossett, J. M. *Appl. Environ. Microbiol.* 1989, 55, 2144-2151.
- (12) de Bruin, W. P.; Kotterman, M. J. J.; Posthumus, M. A.; Schraa, G.; Zehnder, A. J. B. *Appl. Environ. Microbiol.* 1992, 58, 1996-2000.
- (13) Fathepure, B. Z.; Nengu, J. P.; Boyd, S. A. *Appl. Environ. Microbiol.* 1987, 53, 2671-2674.
- (14) Fathepure, B. Z.; Boyd, S. A. *FEMS Microbiol. Lett.* 1988, 49, 149-156.
- (15) Fathepure, B. Z.; Boyd, S. A. *Appl. Environ. Microbiol.* 1988, 54, 2976-2980.
- (16) Mohn, W. W.; Tiedje, J. M. *Microbiol. Rev.* 1992, 56, 482-507.
- (17) Holliger, C.; Schraa, G.; Stams, A. J. M.; Zehnder, A. J. B. *Appl. Environ. Microbiol.* 1993, 59, 2991-2997.
- (18) Gantzer, C. J.; Wackett, L. P. *Environ. Sci. Technol.* 1991, 25, 715-722.
- (19) DiMarco, A. A.; Bobik, T. A.; Wolfe, R. S. *Annu. Rev. Biochem.* 1990, 59, 355-394.
- (20) Ljungdahl, L. G. *Annu. Rev. Microbiol.* 1986, 40, 415-450.
- (21) Jablonski, P. E.; Ferry, J. G. *FEMS Microbiol. Lett.* 1992, 96, 55-60.
- (22) Castro, C. E.; Belser, N. O. *Environ. Sci. Technol.* 1968, 2, 779-783.
- (23) Holliger, C.; Schraa, G.; Stupperich, E.; Stams, A. J. M.; Zehnder, A. J. B. *J. Bacteriol.* 1992, 174, 4427-4434.
- (24) Schanke, C. A.; Wackett, L. P. *Environ. Sci. Technol.* 1992, 26, 830-833.
- (25) Gossett, J. M. *Environ. Sci. Technol.* 1987, 21, 202-208.
- (26) DiStefano, T. D.; Gossett, J. M.; Zinder, S. H. *Appl. Environ. Microbiol.* 1992, 58, 3622-3629.
- (27) Zinder, S. H.; Anguish, T.; Lobo, T. *Arch. Microbiol.* 1987, 146, 315-322.
- (28) Balch, W. E.; Fox, G. E.; Magrum, L. J.; Woese, C. R.; Wolfe, R. S. *Microbiol. Rev.* 1979, 43, 260-296.
- (29) Clesceri, L. S.; Greenberg, A. E.; Trussel, R. R. *Standard methods for the examination of water and wastewater*, 17th ed.; American Public Health Association: Washington, DC, 1989; pp 1467.
- (30) Herzberg, G. R.; Rogerson, M. *Anal. Biochem.* 1985, 149, 354-357.
- (31) Vogel, T. M.; Criddle, C. S.; McCarty, P. L. *Environ. Sci. Technol.* 1987, 21, 722-736.
- (32) Kästner, M. *Appl. Environ. Microbiol.* 1991, 57, 2039-2046.
- (33) Barrio-Lage, G.; Parsons, F. Z.; Nassar, R. S.; Lorenzo, P. A. *Environ. Sci. Technol.* 1986, 20, 96-99.
- (34) Suflita, J. M.; Robinson, J. H.; Tiedje, J. M. *Appl. Environ. Microbiol.* 1983, 45, 1466-1473.
- (35) Semprini, L.; Roberts, P. V.; Hopkins, G. D.; McCarty, P. L. *Ground Water* 1990, 28, 715-727.
- (36) Fathepure, B. Z.; Vogel, T. M. *Appl. Environ. Microbiol.* 1991, 57, 3418-3422.
- (37) Belay, N.; Daniels, L. *Appl. Environ. Microbiol.* 1987, 53, 1604-1610.
- (38) Egli, C.; Scholtz, R.; Cook, A. M.; Leisinger, T. *FEMS Microbiol. Lett.* 1987, 43, 257-261.

Received for review January 24, 1994. Accepted January 28, 1994. *

* Abstract published in *Advance ACS Abstracts*, March 1, 1994.

Reductive Dechlorination of Tetrachloroethene by a High Rate Anaerobic Microbial Consortium

Stephen H. Zinder and J. M. Gossett

Departments of Microbiology and Civil and Environmental Engineering, Cornell University, Ithaca, New York

Tetrachloroethene (PCE) and other chloroethenes are major contaminants in groundwater, and PCE is particularly resistant to attack by aerobes. We have developed an anaerobic enrichment culture that carries out reductive dechlorination of chloroethenes to ethene at high rates, thereby detoxifying them. Although the electron donor added to the culture is methanol, our evidence indicates that H_2 is the electron donor used directly for dechlorination. We have recently obtained a culture from a 10^{-6} dilution of the original methanol/PCE culture that uses H_2 as an electron donor for PCE dechlorination. Because the culture can be transferred indefinitely and the rate of PCE dechlorination increases after inoculation, we suggest that dechlorinating organisms in the culture use the carbon-chlorine bonds in chloroethenes as electron acceptors for energy conservation.

— Environ Health Perspect 103(Suppl 5):5-7 (1995)

Key words: bioremediation, PCE, groundwater, dechlorination, anaerobic PCE dechlorination, electron donor

Chlorinated ethenes are widely employed as solvents and chemical feedstocks. Not surprisingly, groundwater contamination by chlorinated ethenes has become a serious problem in the industrialized world. A 1984 survey of water supplies in the United States listed tetrachloroethene (PCE), trichloroethene (TCE), and the three dichloroethene (DCE) isomers as the five most frequently found contaminants, other than trihalomethanes (1). These compounds pose a public health concern and are therefore regulated by the 1986 amendments to the Safe Drinking Water Act. The remediation of groundwater contaminated with volatile organic chemicals, including PCE, is typically achieved by groundwater extraction followed by a physical/chemical process to remove the pollutants. This approach can be extremely expensive and inefficient; furthermore, physical/chemical processes (e.g., air-strip-

ping and carbon adsorption) simply transfer the hazard from one part of the environment to another.

In contrast, bioremediation applied *in situ* or in above-ground treatment systems offers the prospect of degrading chlorinated ethenes to environmentally acceptable products in a cost-effective manner. Under aerobic conditions, PCE, which lacks a carbon-hydrogen bond, is generally considered to be nondegradable. TCE is co-metabolized by organisms harboring certain nonspecific oxygenases such as methane monooxygenase and toluene dioxygenase (2). Attempts at stimulating degradation of TCE and less chlorinated ethenes in groundwater aquifers by addition of methane and oxygen have met with some success (3). In general, aerobic biodegradability of chloroethenes is inversely related to their degree of chlorination.

Anaerobic degradation of chlorinated ethenes usually proceeds by a reductive mechanism in which the carbon-chlorine bond undergoes hydrogenolysis (Figure 1). Several investigators have found reductive dechlorination of PCE and TCE by anaerobic, usually methanogenic, habitats and by pure cultures of methanogens and acetogens (2,4). Rates were usually low and dechlorination was incomplete, with vinyl chloride (VC) or more highly chlorinated ethenes persisting. Thus, the net result of

anaerobic metabolism of chlorinated ethenes was to produce VC, a known human carcinogen. Freedman and Gossett (5) were the first to report complete anaerobic reductive dechlorination of chloroethenes to ethene (ETH), which is a plant hormone that is nontoxic to humans. More recently, further reduction of ETH to ethane has been described (6). In general, more highly chlorinated ethenes are more degradable under anaerobic conditions than less chlorinated ones. Since the activities of aerobes and anaerobes against chloroethenes are complementary, there is interest in using sequential anaerobic/aerobic processes to degrade them, as well as other highly chlorinated molecules such as polychlorinated biphenyls.

Catalysis of stepwise reductive dechlorination of PCE to ETH *in vitro* using Ti^{3+} as a reductant was demonstrated using transition metal-containing cofactors such as vitamin B_{12} and the nickel-containing methanogenic cofactor F_{430} (7). The kinetics of dechlorination were first-order for each step, and the rate constant for each step was greater than 10-fold lower than for the previous step so that VC dechlorination to ETH was about 10,000-fold slower than PCE dechlorination to TCE. The results suggest that microorganisms rich in transition metal cofactors, such as methanogens and acetogens, could cat-

This paper was presented at the Conference on Biodegradation: Its Role in Reducing Toxicity and Exposure to Environmental Contaminants held 26-28 April 1993 in Research Triangle Park, North Carolina. Manuscript updated: fall 1994; manuscript received: January 23, 1995; manuscript accepted: February 13, 1995.

We thank our colleagues who have worked with us on this project. This research was supported by the United States Air Force's Armstrong Laboratory, Environmental Quality Directorate, Tyndall Air Force Base, Florida, and by the Cornell Center for Advanced Technology (CAT) in Biotechnology, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the National Science Foundation.

Address correspondence to Dr. S. H. Zinder, Departments of Microbiology and Civil and Environmental Engineering, Cornell University, Ithaca, New York 14853. Telephone (607) 255-2415. Fax (607) 255-3904.

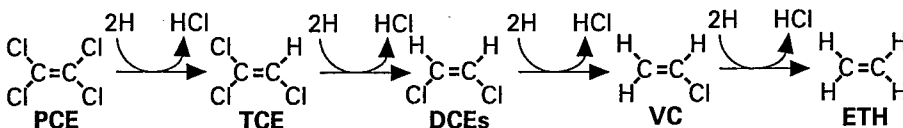


Figure 1. Reductive dechlorination of chloroethenes under anaerobic conditions.

alyze reductive dechlorination by a form of co-metabolism in which reduced forms of these cofactors passively catalyzed the reductions.

The original anaerobic enrichment cultures described by Freedman and Gossett (5) were derived from the anaerobic digester at the Ithaca sewage treatment plant and received nominal PCE doses of 3 $\mu\text{moles/l}$ (about half of this PCE was actually in the headspace); several electron donors appeared to support dechlorination, although methanol (MeOH) appeared to be the most effective. Most of the electron donor was used for methanogenesis, and VC dechlorination to ETH was the rate-limiting step in dechlorination so that considerable amounts of VC persisted.

In a subsequent study (8), we gradually increased the PCE dose to a MeOH-fed culture from 3 $\mu\text{moles/l}$ to 550 $\mu\text{moles/l}$, close to saturation. The MeOH dose was also increased so that it provided approximately twice the electron equivalents needed for complete PCE dechlorination to ETH; a small amount of yeast extract was also added. Increasing the PCE dose actually improved dechlorination such that within 2 days about 80% of the PCE added could be accounted for as ETH with the remainder as VC. Four days of incubation led to 99% conversion to ETH. Furthermore, the culture ceased producing significant amounts of methane, presumably due to inhibition by high PCE concentrations; a fermentation balance showed that the reducing equivalents from methanol not used for PCE reduction (1/3–1/2 of the MeOH added) were used for acetogenesis. The high rates of complete dechlorination of PCE to ETH by the culture were unprecedented as was the high fraction of electron donor used for PCE dechlorination. This culture could be transferred indefinitely, and we have scaled it up to a 6 liter bioreactor (9). We have presented evidence that H_2 is the actual electron donor for dechlorination in the MeOH-fed culture (Figure 2).

More recently (10), we have studied time courses for dechlorination of 550 μmoles PCE added per liter by the MeOH-fed anaerobic enrichment cultures. PCE disappearance was essentially zero-order within the concentration range examined, typically requiring 24 to 36 hr for complete PCE consumption and a specific rate of 4.6 μmoles PCE transformed per milligrams volatile solids per day. Little or no TCE or dichloroethene (DCE) isomers accumulated, and conver-

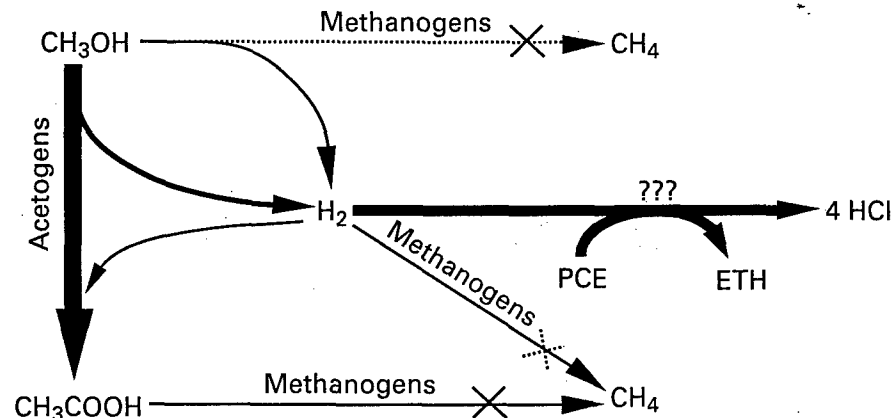


Figure 2. Model for interactions of microbial groups in the MeOH/PCE enrichment culture. Adapted from DiStefano et al. (9).

sion to VC was essentially stoichiometric. Only when most or all of the PCE was consumed were significant amounts of VC converted to ETH. VC conversion to ETH followed first-order kinetics, with a half-life near 17 hr, and ETH was formed stoichiometrically. When VC was added alone to cultures previously fed PCE, it was converted to ETH, with little or no lag, and VC disappearance followed first-order kinetics with a half-life near 17 hr. These results are consistent with inhibition of VC dechlorination by PCE.

This MeOH/PCE culture was capable of dechlorinating all of the other chloroethenes. TCE, *cis*-DCE, and 1,1 DCE were all rapidly and stoichiometrically converted to VC, with subsequent conversion to ETH occurring only when the primary substrate concentrations were low or absent. In contrast, *trans*-DCE disappearance followed first-order kinetics and VC conversion to ETH did not show a lag, suggesting a lack of inhibition of VC dechlorination by *trans*-DCE. It is not clear why substrate utilization followed this pattern, but it should be pointed out that the two chloroethenes for which dechlorination is first-order, VC and *trans*-DCE, lack adjacent chlorines.

We performed a small survey of other haloaliphatics used by the culture and found that the culture could rapidly dehalogenate 1,2-dichloroethane, a common solvent, and 1,2-dibromoethane (ethylene dibromide), a fumigant. In both cases, the primary product was ethene, a typical vicinal reductive dihalo-elimination reaction (4). The culture did not rapidly attack chloromethanes or trichloroethanes, including 1,1,2-trichloroethane, which differs from 1,2-dichloroethane by a single chlorine.

We are presently studying the kinetics of chlorinated ethene utilization more completely, but from these preliminary results, some conclusions can be made. The rates of PCE dechlorination by this mixed culture are three orders of magnitude higher than those obtained for pure cultures of *Methanosarcina* and strain *Desulfomonile tiedjei* (DCB-1) (4), which presumably co-metabolize PCE. The results are also considerably different from those obtained for catalysis by vitamin B₁₂ and cofactor F₄₃₀ (7). These results, along with the substrate specificity observed, suggest that PCE dechlorination is a specific, enzymatically catalyzed process rather than one of passive co-metabolism.

The model presented in Figure 2 makes certain predictions about microbial populations present in the MeOH/PCE culture. It predicts a low population of methanogens and a high population of methanol-utilizing acetogens and H_2 -utilizing PCE reducing organisms. We examined the populations in the culture using MPN analysis (V Tandoi et al., unpublished data). We found that there were low numbers (approximately 104/ml) of H_2 - CO_2 -utilizing methanogens and that methanol-utilizing and acetate-utilizing methanogens were essentially absent. This is of interest because, when the PCE concentration was low, the culture was actively methanogenic, and there were greater than 10^7 methanol-utilizing methanogens present. High numbers ($>10^7$ /ml) of methanol-utilizing Gram positive cocci in chains were found, but there were much lower numbers of H_2 -utilizing acetogenic bacteria. There were high numbers of fermentative heterotrophs, presumably growing on the yeast extract added to the medium, and there were relatively high numbers of sulfate reducing

bacteria, presumably using oxidation products of the sulfide added to the medium. It must be borne in mind that DCB-1 is a sulfate reducer (4).

In MPN dilutions provided with MeOH and PCE, PCE reduction products were found only in dilutions of 10^{-3} or less, and even in those tubes, only low levels of products were detected. In contrast, in tubes provided with PCE and H_2 , PCE reduction occurred in dilutions to 10^{-6} ; PCE was completely consumed in those tubes after a lag of 4 weeks (lags were shorter in lower dilutions), as were subsequent PCE additions. The PCE in those tubes was reduced to VC followed by ETH, which was similar to the original MeOH/PCE enrichment. These results are consistent with our hypothesis (9) that H_2 is the primary electron donor for PCE reduction in the MeOH/PCE culture.

We have transferred this purified H_2 /PCE culture several times, and the rate of PCE dechlorination after transfer increases with time, indicating growth. These purified cultures make neither methane nor acetate, even though there is approximately 0.8 atmospheres of H_2 in the headspace, indicating the absence of methanogens and hydrogenotrophic acetogens. This culture still contains yeast extract-utilizing heterotrophs and sulfate reducers, which are presumably contaminants. The culture apparently requires growth factors present in anaerobic sewage sludge supernatant, and after several feedings of PCE, shows signs of limitation. We

are presently studying this nutrient requirement and are attempting isolation of dechlorinating organisms from the culture.

From the evidence we have obtained, we believe that the culture is using the carbon-chlorine bonds in chlorinated ethenes as electron acceptors for energy conservation in a manner similar to the use of chlorobenzoates by DCB-1. The use of reductive dechlorination reactions for energy conservation has been called chlororespiration (or more generally, halorespiration). The E° values for reductive dechlorination of chloroethenes range from +0.36 to +0.58 V, similar to the nitrate/nitrite couple (+0.43 V), and considerably more favorable than the sulfate/sulfide (-0.22 V) or the CO_2/CH_4 (-0.24 V) couples. Thus there is selective pressure for an anaerobe to evolve the ability to use these compounds as electron acceptors in habitats in which they are present in reasonably high concentrations. It should also be mentioned that Holliger (11) have isolated an organism called *Dehalobacter restrictus*, which grows using H_2 and reduces PCE to *cis*-DCE.

Finally, one may question whether these findings have environmental relevance. Evidence that reductive dechlorination of chloroethenes can be stimulated by electron donors added to groundwater aquifers comes from Major et al. (12), who studied a solvent-contaminated site north of Toronto in which solvents, including PCE and MeOH, were transferred to railroad cars and were apparently spilled at the

points of transfer. At one area of the site, there was a plume of PCE with low concentrations of less chlorinated ethenes present. At another area of the site, PCE concentrations are low, but there are high concentrations of *cis*-DCE, VC, and ETH. Also associated with this area is a compact plume of MeOH, suggesting that this solvent was also spilled in that area. Also associated with the MeOH plume are high concentrations of acetate and chloride. These results suggest that an inadvertent addition of MeOH stimulated reductive dechlorination and acetogenesis *in situ*, similar to the culture we have been studying. More recently, researchers at DuPont (unpublished data) have reportedly stimulated reductive dechlorination of chloroethenes at a contaminated site in Texas, using additions of benzoate as the electron donor.

In summary, we have developed an enrichment culture that converts high concentrations of PCE, such as those found in source zones in contaminated groundwater aquifers, to ETH. We have partially purified the organisms responsible for this dechlorination. A better understanding of the physical, chemical, and physiological requirements of this and other dechlorinating cultures is likely to improve dechlorination of chloroethenes in bioreactors and *in situ*. We are presently studying these factors and are particularly interested in the final step of dechlorination of VC to ETH since this represents true detoxification.

REFERENCES

- Westrick JJ, Mello JW, Thomas RF. The groundwater supply survey. *J Am Water Works Assoc* 76(5):52-59 (1984).
- Ensley BD. Biochemical diversity of trichloroethylene metabolism. *Annu Rev Microbiol* 45:283-299 (1991).
- Semprini L, Roberts PV, Hopkins GD, McCarty PL. A field evaluation of *in situ* biodegradation of chlorinated ethenes. Part 2: Results of biostimulation and biotransformation experiments. *Ground Water* 28:715-727 (1990).
- Mohn WW, Tiedje JM. Microbial reductive dehalogenation. *Microbiol Rev* 56:482-507 (1992).
- Freedman DL, Gossett JM. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl Environ Microbiol* 55:2144-2151 (1989).
- de Bruin WP, Kotterman MJJ, Posthumus MA, Schraa G, Zehnder AJB. Complete biological reductive transformation of tetrachloroethylene to ethane. *Appl Environ Microbiol* 58:1996-2000 (1992).
- Gantzer J, Wackett LP. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ Sci Technol* 25:715-722 (1991).
- DiStefano TD, Gossett JM, Zinder SH. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl Environ Microbiol* 57:2287-2292 (1991).
- DiStefano TD, Gossett JM, Zinder SH. Hydrogen as an electron donor for the dechlorination of tetrachloroethene by an anaerobic mixed culture. *Appl Environ Microbiol* 58:3622-3629 (1992).
- Tandoi V, DiStefano TD, Bowser PA, Gossett JM, Zinder SH. Reductive dehalogenation of chlorinated ethenes and halo-genated ethanes by a high-rate anaerobic enrichment culture. *Environ Sci Technol* 28:973-979 (1994).
- Holliger C, Schraa G, Stams AJM, Zehnder AJB. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl Environ Microbiol* 59:2991-2997 (1993).
- Major DW, Hodgins EW, Butler BJ. Field and laboratory evidence of *in situ* biotransformation of tetrachloroethene to ethene and ethane at a chemical transfer facility in north Toronto. In: *On-Site Bioreclamation—Processes for Xenobiotic and Hydrocarbon Treatment* (Hinchee RE, Olfenbittel RF, eds). Boston: Butterworth-Heinemann, 1991;113-133.

TR Mailing List for EQPP

HQ AETC/CEV
Bldg 661
73 Main Circle
Randolph AFB TX 78150-4549

DTIC
Attn: DTIC-OCP
Bldg 5
Cameron Station
Alexandria VA 22304-6145

HQ AFRES/CEV
155 2nd Street
Robins AFB GA 31098-1635

HQ USAFA/DFSEL
2354 Fairchild Drive, Suite 3A15
USAF Academy CO 80840-6214

HQ PACAF/CEV
25 E Street, Suite D-302
Hickam AFB HI 96853-5412

HQ USAF/CEV
115 Brookley Ave, Suite 1
Bolling AFB DC 20332-5106

AUL/LSD
600 Chennault Circle, Bldg 1405
Maxwell AFB AL 36112-6424

HQ AFMC/CEV
4225 Logistics Ave
Wright-Patterson AFB OH 45433-5006

WL/POTC
1950 5th Street, Bldg 18
Wright-Patterson AFB OH 45433-7251

USAE Waterways Experiment Station
Attn: WES EE-S/John Cullinane
PO Box 631
Vicksburg MS 39180-0631

HQ AFSPACCOM/CEV
150 Vandenberg Street, Suite 1105
Peterson AFB CO 80914-4150

Director
US EPA Robert S Kerr Environmental
Research Lab
PO Box 1198

Cathy Vogel (4)
AL/EQM-OL
139 Barnes Drive, Suite 2
Tyndall AFB FL 32403-5323

Allen Tool
US Army COE
Kansas City District
601 E 12th Street
Kansas City MS 64106-2896

HQ USAFE/CEV
Unit 3050, Box 10
APO AE 09094-3050

HQ AMC/CEV
507 A Street
Scott AFB IL 62225-5022

ANGRC/CEV
3500 Setchet Ave, Bldg 3500
Andrews AFB MD 20331-5157

WL-MNOI - Tech Library
203 W Eglin Blvd, Suite 300
Eglin AFB FL 32542-6843

AFIT/CE
2950 P Street
Wright-Patterson AFB OH 45433-7765

AFIT/LSM
2950 P Street
Wright-Patterson AFB OH 45433-7765

Environmental Quality Division
NAVFAC Code 112
200 Stovall Street
Alexandria VA 22332

HQ ACC/CEV
129 Andrews Street, Suite 346
Langley AFB VA 23665-2769

US Army Research Office
PO Box 12211
Research Triangle Park NC 27709
Ada OK 74821-1198

Dept of the Army
Commander
CERL
PO Box 4005
Champaign IL 61824-4005
Naval Facilities Engineering
Service Center
Code ESC40
5660 Center Drive
Port Hueneme CA 93043-4328

DET 3
Unit 5213
AL
APO AP 96318-5213

Dr. Steve Zinder (5)
Section of Microbiology
Wing Hall
Cornell University
Ithaca, NY 14853

AFCEE/EST
2504D Drive, Suite 3
Brooks AFB TX 78235-5103

NGB/CE
2500 Army Pentagon, RM 2D369
Washington DC 20331-2500

Dr. Jim Gossett (5)
School of Civil & Environmental Engineering
Cornell University
Hollister Hall
Ithaca, NY 14853-3501